

**CELLULAR RESPONSES IN CUTANEOUS SENSITISATION**

**BY**

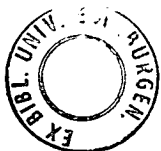
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DECLARATION

I declare that this thesis has been composed by myself and that the work described herein is my own.

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## CONTENTS

	Page
<b>CHAPTER I. GENERAL INTRODUCTION AND INTRODUCTION TO T CELLS</b>	<b>1</b>
General Introduction	1
Introduction to T cells	3
1. Maturation of T cells	3
a. The thymus	3
b. The peripheral lymphoid tissue and circulating cells	9
2. Functions of T cells	13
a. Humoral antibody production	13
b. Cell-mediated immunity	16
c. Regulation of the immune response by T cells	18
Aims of the present studies	21
 <b>CHAPTER II. STUDIES ON THE T CELL PROLIFERATIVE RESPONSE</b>	 <b>23</b>
Introduction	23
Review of the literature	24
1. Contact sensitisation	24
a. The antigen	24
b. Sensitisation	26
c. The histological response	28
2. Sub-populations of T cells and their role in regulating the immune response	34
a. Evidence for positive interactions between T cells	35
b. Evidence for the existence of suppressor T cells	37

	Page
<b>Materials and methods</b>	<b>44</b>
1. Mice	44
2. Irradiation	44
3. Preparation of cell suspensions	44
4. Peripheral blood lymphocytes	44
5. Cortisone treatment	45
6. Anti-theta treatment	45
7. Thymectomies	46
8. Antigens	46
9. Measurement of DNA synthesis	47
10. Statistical analysis	48
<b>Results</b>	<b>49</b>
1. The response of normal mice to different doses of oxazolone	49
2. Timing of the experimental procedures	50
3. Cell transfer experiments demonstrating the response of lymphocytes from different anatomical regions to oxazolone	51
a. Thymocytes	52
b. Cortisone resistant thymocytes	54
c. Lymph node lymphocytes	55
d. Peripheral blood lymphocytes	56
4. Experiment to investigate the effect of thymocytes and cortisone resistant thymocytes on the response of lymph node cells to oxazolone	57
5. The proliferative response of lymphocytes from animals previously immunised with oxazolone	58
6. The effect of adult thymectomy on the ability of lymph node and peripheral blood lymphocytes to transfer the proliferative response to oxazolone	59
7. The proliferative response of thymocytes to ferritin and PVP	62



	Page
<b>Discussion</b>	64
1. Evaluation of the technique used to estimate cellular proliferation	64
2. Parameters of the experimental system	66
3. Kinetics of the T cell proliferative response to oxazolone	68
a. Thymocytes	69
b. Mature T cells	73
4. Experiment to investigate the existence of suppressive or potentiating interactions between T cells, affecting the response to oxazolone	79
5. Factor of immunisation of oxazolone	81
6. Effect of adult thymectomy on the ability of lymphocytes to transfer the proliferative response to oxazolone	83
7. The proliferative response of thymocytes to ferritin and PVP	87
 <b>CHAPTER III. THE ANTIBODY RESPONSE TO OXAZOLONE</b>	 90
<b>Introduction</b>	90
<b>Material and methods</b>	94
1. Mice	94
2. Immunisation of mice	94
3. Preparation of sera	94
4. Measurement of DNA synthesis	94
5. Detection of antibodies to oxazolone	95
a. Coupling of sheep erythrocytes to oxazolone	95
b. Direct haemagglutination technique	96
c. Haemagglutination technique to detect class specific immunoglobulin	97

	Page
<b>Results</b>	98
1. Antibodies in the sera of mice given one or two applications of oxazolone	98
a. Undeveloped antibody	98
b. Developed antibody	99
c. Augmentation of antibodies with anti- $\gamma$ G1, anti- $\gamma$ G2a, anti- $\gamma$ G2b and anti- $\gamma$ A	100
2. The effect of passively administered antibody on the proliferative response of lymph node cells to oxazolone	100
<b>Discussion</b>	102
<b>Summary</b>	109
 <b>CHAPTERS IV AND V. CELL RECRUITMENT DURING THE RESPONSE TO OXAZOLONE</b>	110
<b>Introduction</b>	111
1. Lymphocyte circulation	111
a. The kinetics of T and B cell circulation	111
b. Circulation within the lymph nodes and spleen	114
c. Circulation of activated T and B cells	117
2. Lymphocyte recruitment	121
a. Specific recruitment	122
b. Non-specific recruitment	125
c. Mechanisms of cell recruitment	128
3. Labelling techniques for studying cell localisation	133
a. $\beta$ Emitters	134
b. $\gamma$ Emitters	136
<b>Materials and methods</b>	140

1. Mice	140
2. X-irradiation	140
3. Cell suspensions	140
4. Oxazolone	141
5. Measurement of DNA synthesis	141
6. Plaquing technique	142
7. Labelling techniques	142
a. $^{75}\text{Se}$ -L-selenomethionine	142
b. $^3\text{H}$ -methionine	145
c. L-4,5- $^3\text{H}$ -leucine	145
8. Experiments utilising rat thoracic duct cells	145
9. Autoradiography of lymphocytes labelled with $^3\text{H}$ -methionine	146
10. Estimation of $^3\text{H}$ -leucine incorporation	147
 CHAPTER IV. USE OF $^{75}\text{SE}$ -SELENOMETHIONINE AS AN ISOTOPIC MARKER FOR CELL LOCALISATION STUDIES. EVALUATION OF THE TECHNIQUE	148
Results	148
A. Parameters of incorporation of the isotope	148
1. Kinetics of incorporation	148
2. Incorporation into the TCA insoluble cellular fraction	149
3. Elution of the isotope	150
4. Incorporation and elution of isotope by tumour cells	151
5. Incorporation of isotope by different lymphocytes	151
6. Autoradiography of cells labelled with $^3\text{H}$ -methionine	152

B.	Reutilisation studies	153
1.	Localisation of supernatant counts	153
2.	Localisation of cells killed by freeze-thawing	154
3.	Localisation of allogeneic cells	155
4.	Localisation of semi-allogeneic lymphocytes	156
C.	Studies on the immunocompetence of labelled cells	157
1.	Localisation and proliferation of labelled cells	157
2.	Ability of labelled spleen cells to transfer a primary humoral response to sheep red cells	158
D.	Localisation of labelled cells from various sources	159
1.	Lymph node lymphocytes	159
2.	Cortisone resistant thymocytes	160
3.	Thymocytes	160
4.	Rat thoracic duct cells	161
5.	Localisation of <sup>51</sup> Cr-labelled spleen cells	162
	Discussion	164
1.	The isotope	164
2.	The labelling technique	165
3.	Incorporation of <sup>75</sup> Se by different cell populations	166
4.	Elution of label	167
5.	Reutilisation studies	169
6.	Immunocompetence of labelled cells	174
7.	Localisation of labelled cells from various sources	176
a.	Localisation of cells in lymph nodes and spleen	177
b.	The significance of activity recovered from tissues other than the lymph nodes and spleen	180

	Page
8. Localisation of rat thoracic duct cells	187
9. General comments	187
 CHAPTER V. CELL RECRUITMENT DURING THE RESPONSE TO OXAZOLONE	190
Results	190
1. Experiment utilising $^{75}\text{Se}$	191
2. Experiment Utilising $^3\text{H}$ -leucine	194
Discussion	196
1. The effect of cellular proliferation on cell recruitment	197
2. The kinetics of cell localisation to lymph nodes in oxazolone-stimulated mice	199
 ACKNOWLEDGEMENTS	203
 REFERENCES	204

### SUMMARY

The existence of sub-populations of T cells, distinguished by their maturity, membrane characteristics and functions, has been established, and an introduction to T cell heterogeneity is provided in Chapter I. T cell proliferation is an early event in many immune responses. It is reasonable to assume that any mechanism which regulates the magnitude of T cell proliferation may affect the overall outcome of the immune response, in terms of the types of effector cells produced. In these studies the antigen oxazolone (a potent stimulator of T cell proliferation) is used to discover possible factors that may regulate the proliferative response.

In Chapter II information is presented about the antigen oxazolone and the cellular events that occur after its cutaneous application. The experiments reported in this chapter show that T cells can transfer adoptively the DNA synthetic response to oxazolone into irradiated recipients (measured by uptake of  $^{125}\text{IUdR}$ ) and this system was used to investigate the kinetics of the response. Mature T cells produced cell for cell a greater response than do thymocytes. However, regardless of the source of T cells, a characteristic pattern of response was revealed where peak DNA synthesis occurring on days 3, 4 or 5 could not be elevated by increasing the dose of cells. Thus a homeostatic mechanism appears to regulate the magnitude of the T cell response. Experiments failed to implicate any sub-population of T cells in regulating the response, and the possible existence of other mechanisms is discussed.

In Chapter III the production of antibodies to oxazolone of classes  $\gamma_M$ ,  $\gamma_G$ ,  $\gamma_A$ , and sub-classes  $\gamma_{G1}$ ,  $\gamma_{G2a}$  and  $\gamma_{G2b}$  in the sera of mice given one or two applications of oxazolone was investigated. The possibility that T cell proliferation may be regulated by antibody production was investigated by studying the effect of passively administered hyperimmune serum on the proliferative response. It was found to have no demonstrable effect.

Antigen-mediated recruitment of circulating cells is recognised as being an early event in the initiation of an immune response and an account of cell traffic is given in the Introduction to Chapter IV and V. The relationship between cell proliferation and cell recruitment was studied and the possibility that the regulation of T cell proliferation may be mediated by altering cell traffic was investigated in Chapters IV and V. In Chapter IV a new technique for investigating cell localisation is described, using  $^{75}\text{Se}$ -L-selenomethionine ( $^{75}\text{Sem}$ ) a  $\gamma$ -emitting isotope, as a radioactive marker for lymphocytes. The technique is evaluated within the context of the problems associated with using radioactive isotopes as markers for lymphocytes. The advantages of  $^{75}\text{Sem}$  are the high levels of incorporation that can be achieved and the low levels of in vivo reutilisation and absence of any adverse effects on lymphocyte immunocompetence.

The studies in Chapter V revealed that cutaneous application of oxazolone produces an accumulation of labelled cells in the regional lymph nodes 24-48 hours after application, with a concomitant decrease in the recruitment of cells in the contralateral nodes. Cell traffic to draining and contralateral nodes returned to normal after 4 to 5 days.

The magnitude of cell recruitment was independent of DNA synthesis occurring concurrently in the stimulated node. Thus the factors that regulate the proliferation of T cells did not appear to involve or be mediated via an alteration in cell recruitment. These results are discussed in terms of current concepts concerning the mechanisms that control recruitment of cells in antigenically stimulated lymphoid tissue.



## CHAPTER 1

### GENERAL INTRODUCTION AND INTRODUCTION TO T CELLS

#### General introduction

The adult mammalian immune system depends on interactions between two interdependent components, the circulating immunological tissues, blood and lymph, and the fixed immunological organs, the spleen and lymph nodes, bone-marrow and thymus. The blood and lymph contain antigen, immunocompetent cells and their precursors, and antibody. The immunological viability of this circulating component is dependent on replenishment by the bone-marrow and thymus, and filtration through the spleen and lymph nodes. These structures are reticular meshworks which physically and biologically filter and change the composition of the fluids passing through them. They sequester antigen and immunocompetent cells, provide sites for their interaction, provide a suitable environment for the consequent proliferation and differentiation of activated lymphocytes, and allow the release of the end products able to effect an immune response.

Mammalian blood and lymph contain a variety of leucocytes; lymphocytes and their precursors, monocytes, granulocytes and platelets. These all play a role in defence mechanisms, but the essential unit responsible for specific immunity is the lymphocyte. The outstanding feature of the lymphocyte that distinguishes it from non-specific defence mechanisms is its ability to recognise specific antigenic structures, to become sensitised and produce a heightened secondary response to

that antigen.

Lymphocytes are a heterogeneous population with regard to their membrane characteristics, distribution and function. The ultimate origin of lymphocytes are the haemopoietic stem cells, that is, cells able to differentiate into specialised blood cells and with the capacity for extensive self-renewal (Lajtha, 1966; Barnes & Loutit, 1967a; Caffrey-Tyler & Everett, 1966). The first haemopoietic stem cells of avian and mammalian embryos are found in the yolk sac (Moore & Owen, 1967a) and later in the foetal mammalian liver (Moore & Metcalf, 1970) and in the adult bone-marrow (Barnes & Loutit, 1967a, 1967b). Cells from these organs are seeded via the blood and their differentiation to more mature cells is directed along certain pathways by the inductive influence of the particular environment in which the cells lodge (Moore & Owen, 1967a, 1967b; Metcalf & Moore, 1971). The inductive influence is provided by the 'primary lymphoid organs' (Miller, 1966) which consist of the thymus gland in mammals and birds and the bursa of Fabricius in avian species.

Classically, the vertebrate immune response was described as being of two types, characterised by the production of either humoral antibody or the production of cell-mediated immunity. This dichotomy can be reconciled in terms of differential contribution by cells which differentiate in the thymus or T cells and cells which differentiate in the absence of the thymus or B cells (Roitt et al, 1969). T cells are responsible for initiating cell-mediated immunity and B cells are responsible for the major production of antibody. The majority of

immune responses require the participation of both T and B cells.

The major portion of these studies is concerned with the kinetics and regulation of the T cell proliferative response to the antigen oxazolone. T cells are a heterogeneous population and their maturation and various functions are described below.

### Introduction to T cells

#### 1. Maturation of T cells

##### (a) The Thymus

The thymus is the organ with the highest rate of production of lymphocytes which takes place almost entirely in the thymic cortex, the medullary lymphoid cells exhibit little or no mitotic activity (Metcalf, 1964). In several studies tritiated thymidine was injected into the thymus and after one pulse, many large and medium sized lymphocytes were labelled, but only a few small lymphocytes were labelled. (Metcalf, 1966; Metcalf & Wiadrowski, 1966; Borum, 1968). These results, suggesting that large lymphocytes differentiate to produce small thymocytes accords with the model of cortical lymphopoiesis proposed for the rat thymus by Sainte-Marie and Leblond (1965). Differentiation of large thymocytes to small thymocytes is accompanied by movement of cells from the cortex to the medulla (Borum, 1968; Weissman, 1973). The total cortical population appears to be replaced every 3-4 days (Metcalf & Wiadrowski, 1966; Metcalf, 1966). As the size of the adult thymus remains relatively constant, this rapid renewal

must be balanced by an equally rapid loss through either cell death or migration.

There is good evidence to show that cells migrate from the adult thymus to the peripheral lymphoid tissue. Indirect evidence was provided by experiments where thymectomised mice were restored to immunocompetence by giving them chromosomally marked thymus grafts. Cells of thymic origin were found in peripheral lymph node tissue (Miller, 1962; Miller & Osoba, 1963; Harris et al., 1964; Davies et al., 1966; Leuchars et al., 1964). More direct evidence was provided by labelling thymocytes in situ with the tritiated precursors of DNA and RNA, and following the subsequent appearance of labelled cells in the spleen and lymph nodes of guinea-pigs (Nossal, 1964) and rats (Weissman, 1967). There appears to be some homeostatic mechanism which regulates the rate of outflow of thymocytes from the thymus. For example, Metcalf (1966) grafted mice with 12-48 thymus grafts, injected tritiated thymidine and found the same percentage of labelled cells in peripheral lymph nodes as found in normal mice given only intrathymic thymidine. There is evidence for a population of cells that under normal conditions never leaves the thymus (Elliot, 1973). There is very little evidence of cell death in the thymus, but it is thought that some proliferating cells die (Metcalf, 1966). The function of the intense lymphopoiesis within the thymus could be to allow diversification of cells with regard to their antigen receptors, possibly accompanied by destruction of self-reactive cells (Burnet, 1962).

The origin of cells within the thymus is undoubtedly extrinsic.

Experiments have been performed using chicken chimeras repopulated with different cell populations which can be chromosomally distinguished by the male ZZ or female ZW chromosomes. Thus chicken embryos were parabiosed at the yolk sac stage and found to have a high level of thymic chimerism, and thymic grafts developing on chorioallantois became populated by host cells (Moore & Owen, 1967c). Thymic rudiments from chick and mouse embryos of various ages were removed and their ability to become lymphoid was tested in vitro, the results suggested that stem cells enter the chick thymus at about day 11 (Owen & Ritter, 1969). Bone-marrow cells, distinguished by the mouse chromosome marker T6T6 were found to repopulate the thymus of lethally irradiated syngeneic recipients (Ford & Micklem, 1963) and the shielded thymus of part-body irradiated mice (Ford et al., 1966).

Cells within the thymus differentiate and 'mature' to form thymocytes characterised by their immunocompetence and ability to migrate to the peripheral lymphoid tissue. The production of mature thymocytes is accompanied by rearrangement of surface components distinguished using alloantisera, and can be conveniently described as occurring in two stages;

- (1) The maturation of cells which have recently migrated to the thymus to immature thymocytes.
- (2) The maturation of immature thymocytes to mature thymocytes.

### 1. Development of immature thymocytes

Murine stem cells from foetal yolk sac migrate into the rudimental thymus at about 11 days of gestation (Owen & Ritter, 1969). At this stage they have no theta antigen or thymus - leukaemia (TL) antigen but by 18 days the majority are killed by anti-theta serum and 61% by anti-TL serum (Owen & Raff, 1970). This change is accompanied by cellular proliferation and the cells differentiate to small lymphocytes (Owen & Raff, 1970). The experiments were performed by culturing 14 day thymic rudiments in isolation on chick chorioallantois, thus eliminating the possibility that new cells carrying theta and TL antigen were entering the thymic rudiment. Therefore foetal cells acquire theta and TL antigen in the thymus. There is circumstantial evidence indicating that bone-marrow cells also develop these antigenic characteristics. Bone-marrow cells do not carry theta (Reif & Allen, 1964; Aoki et al., 1969) or TL antigen (Aoki et al., 1969), they have however been shown to become TL positive under thymic influence. Thus thymic grafts of C57Bl/6 strain (TL negative) were implanted under the kidney capsule of thymectomised (A X C57Bl/6)  $F_1$  hosts (TL positive). The grafts were repopulated by host cells which were TL negative on day 11, but after 4 days in the thymus they became TL positive (Schlesinger & Harwitz, 1968). The possibility that TL positive cells were entering the graft between days 11-15 was not excluded.

### ii. Development of mature thymocytes

A population of thymocytes migrates to the peripheral lymphoid tissue where it eventually exhibits immunocompetence and the properties of secondary lymphoid tissue and T cells. Such properties include the

ability to recirculate and certain alloantigenic surface characteristics. Lymph node cells have little theta, no TL, but heavy representation of H2 antigen (Reif & Allen, 1964; Aoki et al., 1969). This contrasts to the high levels of theta and TL found in the majority of thymocytes. Thymocytes were injected into lethally irradiated syngeneic hosts, the spleens were harvested and were found to have alloantigen representation identical to that found in lymph nodes (Raff & Owen, 1970; Raff, 1971). Thymocytes of A strain mice (TL positive) were shown to lose their TL antigen in the periphery (Lance et al., 1970). It is theoretically conceivable that thymocytes acquire these properties while in the periphery, but experiments suggest that at least some are acquired within the thymus. For example, it has been shown that thymocytes in the thymus of a TL positive strain, lose their TL antigen while in the thymus and gain immunocompetence, by experiments that concentrated the cells able to perform graft versus host activity from TL positive thymuses by use of anti-TL sera (Leckband, 1970,1971). The possibility that mature T cells able to mediate a graft versus host response were entering the thymus was excluded by Ritter (1971) who showed development of graft versus host activity in embryonic thymus cultured in diffusion chambers.

Another method of isolating an intrathymic cell population which displays immunocompetence is to administer cortisone acetate (2mg/mouse) 2 days before harvesting the thymus (Cohen et al., 1970). About 95% of the thymocytes are destroyed (Cohen et al., 1970), the surviving cells are found in the medulla (Ischidate & Metcalf, 1963; Warner, 1964) and are TL negative (Schlesinger & Golaki, 1967). The cortisone resistant

medullary thymocytes produce graft vs host responses (Blomgren & Andersson, 1969; Cohen et al., 1970; Blomgren & Andersson, 1970), respond to allogeneic cells in vitro (Blomgren & Svedmyr, 1971; Mosier & Cantor, 1971), to PHA in vitro (Blomgren & Svedmyr, 1971) and cooperate with bone-marrow cells to produce humoral responses to sheep erythrocytes, bovine serum albumin, ovalbumins and NIP (Andersson & Blomgren, 1970). These results are substantiated by Warner's study (1964) which demonstrated that immunocompetence of the chicken thymus resided solely in the medulla. As thymic cortical cells divide they appear to move into the medulla (Borum, 1968) and cortisone resistant thymocytes arise from cortisone sensitive cortical cells (Weissman, 1973). There is evidence linking cortisone resistant thymocytes with circulating cells and peripheral T cells.  $^{51}\text{Cr}$  labelled cortisone resistant thymocytes migrate to lymph nodes in higher numbers than thymocytes (Blomgren & Andersson, 1972; Lance et al., 1970), cortisone resistant cells are TL negative and TL negative cells behave as recirculating cells (Raff, 1971). The "cell volume distribution" of cortisone resistant thymocytes is similar to that of a normal lymph node population (Blomgren & Andersson, 1969).

Cortisone resistant thymocytes thus represent a population of thymocytes that have acquired some maturity, they are not however identical to either lymph node T cells or spleen cells. Results suggest that cortisone resistant thymocytes are a heterogeneous population and that some require 4 or 5 days in the periphery before showing lymph node T cell kinetics to histocompatibility antigens (Mosier & Cantor, 1971; Cantor & Mosier, 1972). This may explain why cortisone resistant



thymocytes do not synergise with thymocytes to produce splenomegally as do lymph node cells, and why spleen cells, when injected into  $F_1$  recipients produce a peak mortality of the recipients 31 days after injection, 4 days earlier than do cortisone resistant thymocytes (Tigelaar & Asofsky, 1973). Tigelaar and Asofsky (1973) suggested the existence of a cortisone sensitive immunocompetent thymocyte to explain the latter observation. This is supported by the fact that although cortisone resistant thymocytes account for 5% of total thymocytes, cell for cell they are only six times more active in T-B cell collaboration (Andersson & Blomgren, 1970) and ten times more active in graft vs host responses (Blomgren & Andersson, 1969; Tigellar & Asofsky, 1973). The existence of cortisone sensitive circulating T cells is also implied by the fact that short lived peripheral T cells are more susceptible to cortisone than long-lived peripheral T cells (Miller & Cole, 1967; Esteban, 1968). Thus while providing a useful tool to concentrate immunocompetent thymus cells, cortisone may not reflect any physiological division of thymocytes and may indeed exclude a mature T cell population and be heterogeneous with regard to the maturity of the thymocytes it does represent.

(b) The peripheral lymphoid tissue and circulating cells

The peripheral lymphoid tissue consists of circulating immunocompetent lymphocytes able to lodge in lymphoid tissue such as the spleen and lymph nodes and respond specifically to antigen by the production of humoral antibody or the effectors of cell-mediated immunity.

The life-span of about 90% of circulating cells in the rat has been calculated as being 2-3 months (Little et al., 1962; Everett and Tyler-Caffrey, 1967). Lymphocytes circulate from the blood through the lymph nodes, through the lymphatics and via the thoracic duct back to the blood (Gowans & Knight, 1964). The route taken by small lymphocytes from the blood to the lymph nodes is through or between the endothelial cells of the post-capillary venules. Electron microscope studies have shown these cells apparently passing through the cytoplasm of the endothelial cells (Marchesi & Gowans, 1964), although more recent evidence suggests the lymphocytes may move between the cells (Schoefl, 1972; Claesson et al., 1971). Lymphocytes are thought to leave the nodes primarily through the efferent lymphatic channels (Hall & Morris, 1964; Gowans & Knight, 1964). The route of cell migration through the spleen is not definitely established. Cells migrate from the blood to the periarteriolar sheaths by passing through gaps in the lining of the endothelial walls of the marginal sinus (Gold-Schneider & McGregor, 1968a) and probably exit via the splenic vein.

The majority of thoracic duct cells are T cells, for about 85% of thoracic duct cells are sensitive to anti-theta serum (Raff, 1969; Raff & Wortis, 1970; Miller & Sprent, 1971a), a treatment which in the presence of complement specifically lyses T cells (Raff & Wortis, 1970; Schlesinger & Yron, 1970). The majority of thoracic duct cells migrate to the paracortex of lymph nodes, the interfollicular areas of Peyer's patches and the periarteriolar sheaths of the spleen. These areas have been termed 'T dependent' areas (Parrott et al., 1966). The thoracic duct also contains B cells (Ellis, 1969; Howard, 1972a, 1972b;

Mandel et al., 1973; Sprent, 1973; Sprent and Basten, 1973) B cells migrate preferentially to the T independent areas of lymph node and spleen (Parrott & de Sousa, 1971; Howard et al., 1972).

There is a fairly large body of evidence to suggest that circulating T cells are not derived directly from the thymus, but indirectly, probably as a result of maturation of newly formed T cells in the periphery. Maturation could be driven by a thymic hormonal factor (Stutman et al., 1969) or by antigen (Cantor, 1971). These possibilities are not mutually exclusive. Miller and Mitchell (1969) found that thymus cells given to lethally irradiated mice produced, in response to sheep red cells, large pyronophilic cells which gave rise to a progeny of smaller lymphocytes. These behaved as recirculating lymphocytes when injected into normal recipients. Similarly, Sprent & Miller (1971, 1972b) injected thymus cells labelled with tritiated thymidine into  $F_1$  recipients. These thymocytes proliferated in response to alloantigens and 4-5 days later labelled cells appeared in the thoracic duct. These cells were specifically cytotoxic as shown by their ability to reject grafts in vivo, and lyse tumour cells in vitro (Sprent & Miller, 1971, 1972c).

Recirculating cells are essential to maintain the integrity of the immune system. There are two bodies of evidence to support this contention;

a) part of the immunodeficiency caused by neonatal thymectomy can be attributed to the concomitant decrease in circulating cells. Neonatal

thymectomy is associated with a depression in the ability of animals to undertake a cellular immune response such as homograft rejection or delayed hypersensitivity (Arnason et al., 1962; Miller, 1961) and also with a depression in some humoral responses (reviewed in Miller & Osoba, 1967). The concomitant decrease in the number of circulating cells was discovered by measuring the number of lymphocytes that could be mobilised within 48 hours of draining the thoracic duct of normal and neonatally thymectomised mice. In this way it was found that  $8.6 \times 10^7$  cells were produced from a normal mouse compared to  $2-3 \times 10^6$  cells from a thymectomised animal (Miller & Mitchell, 1967; Agnew, 1967). This evidence is corroborated by work showing that the spleen and lymphoid tissues of rats which have undergone chronic thoracic duct drainage are identical to the spleen and lymphoid tissue of rats which have undergone neonatal thymectomy (Goldschneider & McGregor, 1968a). Thymectomised mice can be restored to immunocompetence by the intravenous injection of thoracic duct cells (Agnew, 1967; Miller et al., 1967a; Sprent & Miller, 1971) and cells from neonatally thymectomised mice do not restore immunocompetence (Agnew, 1967).

b) The functional capacity of the lymphoid compartment of lymph nodes and spleen has been shown to depend substantially on the entry of cells from the pool of recirculating lymphocytes, rather than on the cells resident within the node. Hall and Morris (1964) showed that 2,000 R irradiation did not impair the haemolysin response of the locally irradiated sheep popliteal lymph node. The node must therefore have responded by the recruitment of recirculating cells. Local heavy irradiation of the spleen has also been shown not to damage the

haemolysin response (van den Broek, 1971; Simić & Petrović, 1970).

The elegant experiments of Ford and Gowans (1967) demonstrated that the haemolysin response of a spleen, isolated and perfused in vitro, was proportional to the number of cells circulating through the spleen. Their results suggested the existence of a compartment of lymphocytes within the spleen representing perhaps 20% of the spleen population which is critical for the immune response and is dependent on replenishment by the blood.

It is possible to postulate why a recirculating source of immunocompetent cells is more efficient than a sessile population. For example, selective theories of immunity suggest that only a small proportion of lymphocytes are able to react to a particular antigen. Cell traffic would thus allow a much larger number of eligible cells to be made available for induction allowing antigenic selection from the whole pool of recirculating cells, rather than only those present in a certain location (Ford and Gowans, 1967). It is also possible that cells moving through a lymph node or spleen may facilitate contact between antigen and immunocompetent cells. The mechanism of cell recruitment during an immune response is discussed in Chapter IV and V.

## 2. Functions of T cells

### (a) Humoral antibody production

Neonatal thymectomy is associated with defects in the ability to undertake certain antibody responses (Miller & Osoba, 1967) such as those to heterologous erythrocytes and heterologous proteins. The

IgG phase is more severely depleted than the IgM (Miller et al., 1967b; Taylor & Wortis, 1968). There are some antigens that when administered to T cell deprived animals elicit as good a response as in normal animals. These antigens appear not to require the presence of the thymus for an optimal response and are termed T independent. A common characteristic of T independent antigens is that structurally they are composed of repeating identical units, examples of such antigens are SIII pneumococcal polysaccharide (Humphrey et al., 1964; Howard et al., 1971), endotoxin from E. coli (Möller & Michael, 1971; Andersson & Blomgren, 1971), polyvinylpyrrolidone (Andersson & Blomgren, 1971), and polymorised flagellin from Salmonella adelaide (Foldmann & Basten, 1971).

Evidence suggesting synergy between T cells and B cells in the production of antibody to T dependent antigens arose from the results of several types of experiment;

1. Irradiated or thymectomised and irradiated mice when reconstituted with mixtures of thymus cells and marrow cells produced more antibody against sheep erythrocytes than could be accounted for by the sum of the activities alone (reviewed by Clamen & Chaperon, 1969).

11. Davies et al. (1966, 1967) used mice which had been adult thymectomised, lethally irradiated bone-marrow reconstituted and given thymus grafts with slightly different immunogenicity to the bone-marrow donors. Thirty days after thymus grafting, the mice were immunised with sheep erythrocytes and their spleens transferred into irradiated recipients previously immunised against either the thymus or the bone-

marrow donors. The recipients immunised against the thymus donor produced a haemolysin response, while those which had been immunised against the bone-marrow donors produced no antibody. These results intimated that the antibody producing cells were bone-marrow derived but did not exclude the possibility that B cells had been 'processed' by the thymus during the 30 days between thymus grafting and immunisation.

iii. It was found that spleen cells and sheep erythrocytes when injected into irradiated recipients produced antibody forming cells in the spleen detected as 'haemolytic foci'. Thymus cells alone produced no foci,  $10^6$  thoracic duct cells gave 5 haemolytic foci, bone-marrow cells alone gave no foci, but a combination of  $10^6$  thoracic duct cells and  $10^7$  bone-marrow cells, although having no effect on the number of foci, increased the number of antibody forming cells per focus. Unequivocal evidence that the antibody was derived from bone-marrow cells and not thymus cells was obtained by reconstitution of thymectomised mice with semi-allogeneic thymocytes of thoracic duct cells and identification using alloantisera (reviewed by Miller & Mitchell, 1969 and Miller, 1972).

It is established that T cells are essential to allow B cells to produce antibody against certain antigens. A three cell interaction is probably the minimum number of cell types involved in the production of antibody, since macrophages are evidently necessary to either present antigen to B cells or perhaps process it into an immunogenic form (reviewed by Feldmann & Nossal, 1973). Another aspect of the role of

T cells in antibody production is that the helper T cell recognises the 'carrier specificity' of the antigenic molecule and allows and augments the B cell response which is directed against the hapten (Mitchison, 1971; Miller, 1972).

(b) Cell-mediated immunity

Neonatally thymectomised mice are severely depleted in their ability to reject allogeneic skin grafts or tumours, to give delayed hypersensitivity responses to tuberculin (Arnason *et al.*, 1962; Miller & Osoba, 1967) and to give contact sensitisation responses to low molecular weight chemicals such as oxazolone (Parrott & de Sousa, 1969; Parrott *et al.*, 1970). The exact role of the T cell in these phenomena can be considered as an initial antigen specific proliferative response, followed by the activity of effector cells which were possibly produced by the initial proliferation.

The proliferative response of T cells to antigen was first shown by the use of thymectomised, irradiated, bone-marrow reconstituted mice which were implanted with a chromosomally marked thymus graft. Cells of thymic origin were found in the peripheral lymphoid tissue and were observed to proliferate in response to sheep erythrocytes (Leuchars, *et al.*, 1966; Davies *et al.*, 1966) and a skin homograft (Davies *et al.*, 1966). Thymocytes and thoracic duct lymphocytes when injected into irradiated recipients proliferate in response to a variety of antigenic stimuli. Proliferation has been measured by the incorporation of tritiated thymidine (Sprent & Miller, 1971, 1972a) and <sup>125</sup>I-iodinated deoxyuridine (Gershon & Hencin, 1971) and by counting mitotic indices



in chromosome chimeric mice (Davies et al., 1966). Thus thymocytes proliferate in response to histocompatibility antigens (Sprent & Miller, 1971, 1972a; Gershon & Liebhaver, 1972), sheep erythrocytes (Gershon & Hencin, 1971; Kruger & Gershon, 1972; Davies et al., 1969a; Carter et al., 1969), ferritin, polymerised flagellin, keyhole limpet haemocyanin, Brucella abortus (Kruger & Gershon, 1972), and Salmonella flagellin (Davies et al., 1970). In descending order the magnitude of the proliferative response to various antigens was found to be as follows; oxazolone, sheep red cells, bovine gamma globulin, bovine serum albumin, Salmonella flagellin (Carter et al., 1969). Thus the extent of T cell proliferation varies in response to different antigens.

The immunological significance of T cell mitotic activity is not clearly established. For example, whether proliferation represents a maturational procedure necessary to produce effector cells, or whether it represents simply amplification of effector cells or whether mitotic activity is necessary to produce 'helper T cells' is unknown. The products of T cell proliferation have one or more of a variety of characteristics;

- i. They show helper activity towards the antigen that initiated their proliferation.
- ii. They may show an increased ability to proliferate in response to further contact with the original antigen.

iii. They have the property of diapedesis and can thus invade skin grafts or areas where a contact sensitising agent has been administered (Koster et al., 1971; Asherson et al., 1973a).

iv. They have the ability to recirculate (Sprent & Miller, 1972b; Miller & Mitchell, 1970).

v. They possess cytotoxic properties; cytotoxic properties include the mechanisms whereby allografts are specifically rejected in vivo (Sprent & Miller, 1972c), specific cytotoxicity against  $^{51}\text{Cr}$  labelled mastocytoma cells in vitro (Sprent & Miller, 1972c) and non-specific cytotoxicity against  $^{51}\text{Cr}$  labelled mastocytoma cells (Asherson et al., 1973a). The rejection of allografts involves other leucocyte types such as macrophages and granulocytes, the release of soluble mediating factors such as migration-inhibition-factor (MIF), chemotactic factors and transfer factor (Waksman & Colley, 1971). Much of the histological appearance of delayed hypersensitivity is mediated by pharmacological substances released from cells other than T cells (Turk, 1967a).

The question whether different T cell populations mediate these various functions, or whether the same T cell can perform a variety of functions depending on its environment and mode of stimulation is unresolved.

#### (c) Regulation of the immune response by T cells

There is compelling evidence to suggest that T cells, or a population of T cells, play a role in regulating the magnitude and quality of the immune response. Two known features of T cells suggest that they may

include suitable cells for such a function.

(1) B cells require the cooperation of T cells in order to produce antibody to the majority of antigens. The helper function of T cells is carrier specific (Mitchison, 1971). The existence of a system which necessitates the recognition of two antigenic epitopes by different cell populations in order to induce an immune response, provides a sensitive mechanism of regulation.

(2) Unresponsiveness is both more easily induced by low and high doses of antigen, and is more prolonged in T cells than B cells (Taylor, 1969; Chiller et al., 1971).

These two properties suggest that unresponsiveness in the immune system is in most instances mediated at the T cell level. Allison et al. (1971) suggested that T cell unresponsiveness to self antigens prevents potentially self reactive B cells from responding to self-antigens, and any mechanism that overrides the necessity for T cells, such as virus infections, may lead to a breakdown in the control system and result in autoimmunity. Recent experimental evidence suggests the existence of T cell sub-populations that can either augment or suppress the immune response. The responses affected are both humoral and cell-mediated. The evidence is as follows:

1. Examples of T cells augmenting the immune response

- a) The helper T cell which is carrier specific and cooperates with B cells to enable them to produce antibody (reviewed in Transplantation Reviews, 1969).

b) Synergistic interactions between T cells mediating graft vs host responses have been documented. T cells found in the thymus and spleen synergise with T cells found in the lymph node, blood and thoracic duct to produce responses greater than can be accounted for by the sum of the responses (Raff & Cantor, 1971; Cantor, 1972a; Cantor & Asofsky, 1972). In these experiments the graft vs host response was measured by splenomegally or mortality assays. Other experiments have measured the synthesis of DNA by cells which are participating in a graft vs host response and it has been concluded that there are synergistic interactions taking place (Liebhaber et al., 1972; Gershon & Liebhaber, 1972).

#### 11. Examples of T cells suppressing the immune response

a) There is evidence that T cells suppress the formation of allotypic antibody (Herzenberg et al., 1973), antibody to T independent antigens (Baker et al., 1970b), homocytotropic antibody (Okumura & Tada, 1971a, 1971b) and antibody directed against self antigens (Allison et al., 1971).

b) Reports from Gershon and his co-workers suggest synergistic responses between T cells mediating a graft vs host response and negative or suppressive interactions were also suggested (Liebhaber et al., 1972; Gershon & Liebhaber, 1972; Gershon & Liebhaber, 1974).

Gershon (1974) suggests that T cells can respond to signals of the immune response and reply by either enhancing the response or suppressing it, thus returning the response to some optimal level. The possible existence of subpopulations of T cells, or regulatory T cells is

pertinent to the subject of this thesis and the evidence outlined in 1b), 11a), and 11b) above is described in more detail in Chapter II.

#### Aims of the present studies

T cell proliferation precedes many immune responses. The functions of the products of proliferation may be three-fold;

- (1) To mediate T cell cytotoxicity, as occurs in the rejection of allografts or in response to contact sensitising agents.
- (2) To collaborate with antibody producing cells.
- (3) To produce memory cells for both the cytotoxic and humoral responses.

It is apparent that the overall outcome of the immune response, that is the proportion of cells able to collaborate with B cells, able to be cytotoxic or able to give a memory response, may depend on the magnitude of, and cell interactions during, the initial phase of cellular proliferation.

Evidence in the literature suggests the existence of sub-populations of T cells, some of which may have a regulatory role. The aims of the present studies were to investigate the proliferative response of T cells to discover;

- a) whether T cell subpopulations demonstrate the evidence for T cell interactions and whether these interactions could contribute to regulating the magnitude of the response. (Chapter II)

b) antibody is produced in the majority of immune responses, the possible role of B cells and their products in regulating T cell proliferation was investigated (Chapter III).

c) Antigen-mediated recruitment of circulating cells is recognised as being an early event in the initiation of the immune response (Ford & Gowans, 1967). The relationship between cell proliferation and cell recruitment was studied and the possibility that any regulatory role exerted by T cells was mediated by altering cell traffic was investigated (Chapters IV & V).

## CHAPTER II

### STUDIES ON THE T CELL PROLIFERATIVE RESPONSE

#### INTRODUCTION

The antigen chosen to investigate the kinetics of T cell proliferation was 2-ethoxy-methylene-4-phenyl oxazolone (oxazolone). Oxazolone was first introduced by Gell, Harrington and Rivers (1946) and has been shown to be a potent contact sensitising agent (Oort & Turk, 1965) and cause T cell proliferation (Parrott & de Sousa, 1966; Davies *et al.*, 1969b; Carter *et al.*, 1969). The system used to measure cell proliferation was to irradiate mice (900 R) and repopulate them with various doses of lymphocytes obtained from different lymphoid organs. The animals were given oxazolone and cell proliferation was measured 3, 4 and 5 days later by incorporation of  $^{125}\text{I}$ -labelled iodinated deoxyuridine.

The kinetics of the proliferative response were investigated with a view to obtaining some information about:

- (i) the presence of T cell sub-populations,
- (ii) evidence for T cell interactions, that may contribute to regulating the response.

A review of the relevant literature, presenting information about the antigen and T cell sub-populations is provided.

## REVIEW OF THE LITERATURE

### 1. Contact sensitisation

#### (a) The antigen

Application to the skin of any of a large number of low molecular weight chemical substances can sensitise mice, such that the application to the skin of the same substance about a week later produces an inflammatory response with all the features of delayed hypersensitivity. (An account of the histological features associated with delayed hypersensitivity is found in Turk, 1967<sup>a</sup>).

Landsteiner showed contact sensitisation in guinea-pigs to various chloro- and nitro-substituted benzenes (Landsteiner & Jacobs, 1935). He suggested that simple compounds become antigenic by combination with body protein, a view propounded as early as 1907 by Wolff-Eisner (referred to by Eisen & Belman, 1963). The importance of chemical combination with protein became evident from several experiments. For example, there is a correlation between the ability of simple compounds to combine with aniline and their ability to sensitise guinea-pigs (Landsteiner & Jacobs, 1936). Also, erythrocyte stroma conjugated with dinitrofluorobenzene (DNFB) or picryl chloride elicits contact sensitisation when injected with Freund's adjuvant intraperitoneally in guinea-pigs (Landsteiner & Chase, 1941). It was found that 2, 4-dinitrochlorobenzene and similar compounds react with lysine amino groups of epidermal proteins and that 2, 4-dinitrophenyl sulphonyl chloride and 2, 4-nitrophenyl thiocyanate combine with the disulphide bonds of cystine (Eisen &



Belman, 1953). Using DNCB labelled with  $^{14}\text{C}$  and applying it to the skin, it was found that about 20% was excreted in the urine, 70-80% was disseminated in non-cutaneous structures, and the 5% remaining in the skin was exclusively localised in the epidermis (Eisen & Tabachink, 1958).

Benacerraf and Gell (1959a) discovered that the specificity of the cellular delayed response was different from that of the antibody response to proteins conjugated with picryl chloride and oxazolone. Thus they detected delayed hypersensitivity against the protein carrier and antibodies against the hapten. In an attempt to define the 'carrier' produced by painting the chemical directly on the skin, Benacerraf & Gell (1959b, 1961) immunised guinea-pigs with picryl-protein conjugates of heterologous and homologous proteins or pure picryl chloride and challenged with intradermal conjugates or by contact. Immunisation against the heterologous conjugates produced good delayed hypersensitivity against another conjugate, much better than the reaction to contact. Conversely, animals given picryl chloride by contact, gave better contact responses than responses to intra-dermal injections, and intra-dermal injections of homologous proteins decreased the differences. The authors concluded that the picryl group combined in vivo with a fibrous semi-soluble protein or with a cell membrane. Thus the in vivo conjugate involves a much broader based group than only the picryl moiety.

The structure of oxazolone is such that it may combine with the free amino groups of proteins. It does not apparently produce as much macroscopic inflammation as picryl chloride. On the basis of the work

of Benacerraf and Gell it seems reasonable to suppose that the antigenic moiety recognised by the cells which mediate the delayed response to oxazolone consists of oxazolone plus self-protein or altered self-protein. If this supposition is correct, it would possibly be expected that a large proportion of lymphocytes might recognise the altered self-protein, as is the case where about 7% of circulating lymphocytes respond to transplantation antigens (Ford & Atkins, 1972). It is known that lymphocytes proliferate in response to antigen, if the degree of proliferation is accepted as a reflection of the number of antigen reactive cells (a highly speculative proposition) then oxazolone is recognised by a larger number of lymphocytes (able to proliferate) than are sheep red blood cells, bovine gamma globulin or bovine serum albumin (Carter et al., 1969).

(b) Sensitisation

The initial cellular response in the lymph node draining the site of application of a homograft or contact sensitising agent are very similar (Scothorne & McGregor, 1955; Parrott, 1967). Both responses consist of cellular proliferation, mainly confined to days 4-6 after sensitisation, in the paracortex and may be initiated by entry of sensitised circulating lymphocytes or antigen. The relative importance of sensitisation of lymphocytes in the periphery or in the lymph node is unresolved.

An intact afferent lymphatic system, draining the site of sensitisation, is essential for primary rejection of homograft skin (Frey & Werke, 1957; Lambert et al., 1965; Barker & Billingham, 1968). Barker and

Billingham, (1968) demonstrated that the efferent limb of the response is independent of the lymphatics, thus the afferent lymphatics are necessary for sensitisation. This is apparently not so for kidney grafts which can be rejected with only a vascular connection to the body (Hume, et al., 1955; Lavender et al., 1968; Strober & Gowans, 1965).

This has to be reconciled with the observation that certain vascularised sites, such as the cheek pouch of the hamster, enjoy exemption from cell-mediated rejective processes (Billingham et al. 1960). Possibly different organs require different environments to produce sensitisation. Thus blood may normally be a poor vehicle because the putative agent is diluted, but the kidney with a large area of blood contact may negate this disadvantage. On the other hand, skin sensitisation may take place in the parenchyma which requires a rich lymphatic drainage to contact the local node.

That lymphocytes become sensitised in the periphery was postulated by Medawar (1958) and supported by Brent and Medawar (1967), on the basis that peripheral blood lymphocytes participate in cell-mediated immunity. It is difficult to conduct a definitive experiment because the circulating lymphocyte is essential to effect the response as well as possibly initiate it. Similarly the possibility that the antigen or cell debris found in the afferent lymph draining a homograft (Hall, 1967) or medullary sinuses of nodes draining the site of contact sensitisation (deSousa & Parrott, 1969) has initiated central lymphocyte sensitisation cannot be precluded. (de Sousa & Parrott, 1969) investigated the histology of the skin and lymph nodes during the initial response to oxazolone in normal and thymectomised mice. T

dependent lymphocytes were found after 2-4 hours in the skin, but the absence of these cells in the thymectomised mice was not necessarily the cause of their failure to respond. Macrophages containing melanin were found in cortico-medullary junctions in both treated groups, suggesting that skin components had reached the node. It is interesting that germinal centres and plasma cells were present in nodes of thymectomised animals and increased with multiple applications of oxazolone. The possibility that antigen reaches the draining node combined with soluble proteins in afferent lymph is suggested by the findings of Hall and Smith (1971), who painted sheep with isotopically labelled dinitrofluorobenzene. They failed to find isotope associated with cell debris in the afferent lymph but did find it attached to soluble proteins.

(c) The histological response

The response to oxazolone includes two major events:

- (i) The cellular proliferation which occurs in the draining lymph nodes. This is assessed by the appearance of pyroninophilic blast cells or by the incorporation of <sup>125</sup>I-iodinated deoxyuridine (Oort & Turk, 1965; Pritchard & Micklem, 1972).
- (ii) The cutaneous response after challenge. This is measured usually 7 days after sensitisation, by challenging the ear and measuring the increase in its thickness 2-48 hours later.

The nature of these events and their probable interdependence is described below. Oort and Turk (1965) performed histological and

autoradiographical studies on sections of auricular lymph nodes 2-6 days after the ears of guinea-pigs had been painted with oxazolone.

These studies revealed two important points:

- (i) The proliferation of pyroninophilic blast cells was mainly confined to the paracortical zone (that is, the deep cortex) and reached a peak 4 days after stimulation. At this time the paracortex had expanded to occupy one third of the lymph node area in histological sections.
- (ii) A transformation was observed of large  $^3\text{H}$ -thymidine labelled pyroninophilic cells to small labelled lymphocytes between days 4-6. Cells of the plasma cell series were found in the medulla on days 5 and 6.

The histological response to oxazolone appeared similar to the increase in 'tertiary cortical nodules' found in rabbit lymph nodes 4 days after application of a homograft (Scothorne & McGregor, 1955), to the day 4 pyroninophilic cells found in imprints from stimulated lymph nodes (Turk & Stone, 1963) and to the effects of various carcinogens on the draining lymph nodes of mice (Fjelde & Turk, 1965). Subsequent experiments have verified that the peak appearance of pyroninophilic blast cells in response to oxazolone in mice, occurs 4 days after oxazolone (Parrott & de Sousa, 1966; de Sousa & Parrott, 1969) but the peak blast cell response to allografts appears to occur 6-8 days after grafting (Turk, 1967b; Parrott, 1967; Micklem & Brown, 1967).

Scothorne and McGregor (1955) postulated that the large dividing cells are important in mediating graft rejection, although no blast cells were

found at the site of the graft. This paradox was to a certain extent clarified by the demonstration by Gowans (1962) that large pyroninophilic blast cells could transform into small lymphocytes, and this actually occurred in the response to oxazolone (Oort & Turk, 1965).

That cellular proliferation in the paracortex is dependent on the presence of a thymus was clearly established by evidence of two types; first that this area was depleted in neonatally thymectomised (Parrott *et al.*, 1966; Parrott, 1967; de Sousa & Parrott, 1969) and nu/nu mice (de Sousa *et al.*, 1969), and secondly that radiolabelled thymocytes home specifically to these areas (Parrott *et al.*, 1966; Parrott & de Sousa, 1971;) and a thymus graft restored the depleted paracortical areas in nu/nu mice (de Sousa & Pritchard, 1974).

On the basis that the major initial response to oxazolone in the draining lymph nodes occurs in the paracortex, and that cellular proliferation in this area is T dependent, it can be inferred that the cells which respond at this time to oxazolone are T dependent. This hypothesis has been confirmed by comparing the histology of lymph nodes from normal and neonatally thymectomised mice that had been painted with oxazolone. Mice which had been thymectomised lacked a proliferative response in the paracortex (Parrott & de Sousa, 1966; Parrott, 1967; de Sousa & Parrott, 1969) although the histology of the germinal follicles and medullary area were the same as in normal mice and in mice stimulated with pneumococcal polysaccharide S III (de Sousa & Parrott, 1969). The T dependence of the initial proliferative response was also confirmed independently by other

workers. Mice which had undergone adult thymectomy, lethal irradiation and bone-marrow reconstitution were found not to produce a proliferative response to oxazolone measured by counting the number of mitotic cells. Dividing cells were found however in the presence of a thymus graft, and were of thymic origin (Davies *et al.*, 1969). Athymic nu/nu mice produced no proliferative response to oxazolone as assessed by the incorporation of <sup>125</sup>I-iodinated deoxyuridine (Pritchard & Micklem, 1972).

The ability to present the delayed response is also thymus dependent. This is illustrated by the findings that no delayed response was detected in neonatally thymectomised mice (de Sousa & Parrott, 1969), adult thymectomised irradiated mice (Parrott *et al.*, 1970), and athymic nu/nu mice (Pritchard & Micklem, 1972).

The cell interactions and pharmacological agents that produce the inflammation and erythema of a delayed hypersensitivity response are largely unknown. During this process a variety of leucocytes infiltrate the affected area, including cells of the monocyte, polymorph and lymphocyte series. It has been postulated that the pyroninophilic blast cells found in nodes draining the site of application of oxazolone 4 days after oxazolone treatment are the same cells or the precursors of the cells that:

- a) show non-specific cytotoxicity in delayed responses and move to sites of inflammation (Asherson *et al.*, 1973b)
- b) migrate to bone-marrow, where they proliferate and provide a source of sensitised cells to respond to future antigen (Asherson & Zembala, 1973).

Delayed hypersensitivity to oxazolone (Asherson & Ptak, 1968) can be passively transferred by lymph node cells 3-4 days after antigen, by spleen cells 4-5 days after oxazolone, and by peritoneal exudate and bone-marrow cells 7 and 10 days after cutaneous sensitisation (Ptak & Asherson, 1969; Asherson & Zembala, 1973). The ability of lymphoid tissue to transfer delayed hypersensitivity rapidly declines with time using lymph node and spleen cells but persisted with bone-marrow.

Both the macrophage and lymphocyte components of peritoneal exudate cells were able to transfer delayed hypersensitivity (Asherson & Zembala, 1970). Passive transfer was found to be antigen specific and sensitive to anti-theta and complement, and is therefore T dependent (Zembala & Asherson, 1973). The resistance of transfer by peritoneal exudate cells to anti-theta treatment was thought to be due to the presence of theta resistant macrophages.

Indirect evidence that pyroninophilic blast cells produced by oxazolone move non-specifically to sites of inflammation has been produced.

<sup>51</sup>Cr-labelled lymph node cells from immunised donors were injected into syngeneic recipients and homed to inflamed ears in significantly higher amounts than unimmunised lymph node cells (Asherson & Allwood, 1972). These migratory cells were sensitive to anti-theta and complement (Asherson et al., 1973a) and were only produced by 'T dependent' antigens (Asherson & Allwood, 1972). At least some of these cells were blast cells, for a higher proportion of <sup>125</sup>I-iododeoxyuridine labelled (in vitro and in vivo) cells arrived at areas of inflammation than did cells labelled with <sup>51</sup>Cr (Asherson et al., 1973a), and they did not include macrophages (Asherson et al., 1973a).



Proliferation of blast cells in the draining lymph node appears to be associated with the appearance of migratory cells, for mice made tolerant to picryl chloride did not develop lymphocytes that homed to inflamed areas (Asherson & Allwood, 1972) and neither did they give a DNA synthetic response to picryl chloride (Asherson & Barnes, 1973).

As yet there is no direct evidence that blast cells leave the lymph node in intact mice. Evidence from other sources suggest that this is possible. There was an increase in the number of immunoblasts in the thoracic duct lymph of rats 4 days after administration of dinitro-chlorobenzene (Delorme et al., 1969), 4 days after the injection of  $F_1$  recipients with parental cells (Sprent & Miller, 1972b) and 7 days after bacterial infection with Listeria (Koster et al., 1971; McGregor et al., 1971). Cytotoxicity may be a property to be expected from blast cells about to migrate to inflamed regions, and indeed pyroninophilic cells produced<sup>by</sup> oxazolone stimulation were shown to be non-specifically cytotoxic, reaching their peak in cytotoxicity 4 days after oxazolone (Asherson et al., 1973<sup>b</sup>). Blast cells in the thoracic duct which were produced in response to transplantation antigens were found to be specifically cytotoxic (Sprent & Miller, 1972c). Thoracic duct blast cells produced in response to Listeria when injected into syngeneic recipients protected them from Listeria infection (Koster et al., 1971; McGregor et al., 1971). The suggestion that some blast cells move to sites of inflammation is given credence by the findings that some blast cells do not recirculate (Delorme et al., 1969; Sprent & Miller, 1972b; McGregor et al., 1971) and may acquire the ability to extravasate into inflamed tissues (Koster et al., 1971;

Rosentreich et al., 1971). Other types of cells also appear in sites of inflammation and the majority appear to be non-specific and of a series other than the small lymphocyte (McClusky et al., 1963; Najarian & Feldman, 1963; Luberoff & Waksman, 1968a, 1968b; Spector & Willoughby, 1968).

## 2. Sub-populations of T cells and their role in regulating the immune response

The varied functions of effector cells which are produced as a result of the activation of T cells by antigen, may reflect different cell populations. In that instance the question arises whether the sub-populations were present before antigen activation, or only after as a direct consequence of antigen activation. The different functions of effector cells may however denote the presence of one multi-effector cell population, the responses of which depend on either the micro-environment or perhaps the form in which the antigen is presented to it. Most of the experiments that indicate the existence of T cell sub-populations cannot distinguish between the effects of primary T cell sub-populations and populations of activated T cells.

The existence of functional heterogeneity among the T cell population implies the existence of a system that may qualitatively and quantitatively regulate the T cell and perhaps B cell response. Heterogeneity has been detected in situations where there is a positive interaction between T cells, thus boosting the response and negative interactions, thus suppressing the response.

(a) Evidence for positive interactions between T cells

Lymphoid tissues differ in their ability to mediate graft vs host responses measured by the production of splenomegally. The descending order of reactivity of tissues are peripheral blood lymphocytes (PBL), lymph node cells, spleen cells and thymocytes (Cantor & Asofsky, 1970; Tigelaar & Asofsky, 1972). The addition of an exceedingly small number of relatively active cells (such as PBL) to inactive cells (thymocytes) produced a response greater than could be predicted from the sum of their activities (Cantor & Asofsky, 1970; Tigelaar & Asofsky, 1972). Both cell types must be allogeneic to the host (Cantor & Asofsky, 1970) and both are depleted by neonatal thymectomy (Cantor & Asofsky, 1972) or by anti-theta serum and complement (Cantor, 1972b) indicating the T dependence of these cells.

Evidence that the two cell types differ in their properties stems from the differential effects of anti-thymocyte serum (ATS). Thus the graft vs host activity of the spleen was severely damaged by ATS but was restored with small numbers of PBL, and the amplifying ability of PBL was lost more rapidly than their ability to respond independently (Cantor & Asofsky, 1972). Therefore two types of T cell differing in their properties and distribution may be distinguished, (i) the amplifier cells which are found in blood (Cantor & Asofsky, 1972), lymph nodes (Cantor & Asofsky, 1970), and thoracic duct (Cantor, 1971), and which are sensitive to anti-lymphocytic sera (Cantor & Asofsky, 1972), and (ii) the precursor cells which are found mainly in the spleen and thymus and which are resistant to AIS.

This bimodal distribution of T cells is supported by investigations into the properties of cells which form rosettes with sheep red cells (SBC). A population of immune and background rosette forming cells to SBC have been shown to be sensitive to anti-theta and complement (Greaves & Moller, 1970; Bach et al., 1970) and thus were assumed to be T dependent (TRFC). Background TRFC to SBC which are found in the spleen and thymus are sensitive to anti-theta and complement and azothioprine (Bach & Dardenne, 1972), and appear to have only one immunoglobulin light chain on their surface (Greaves & Hogg, 1971). They are eliminated by adult thymectomy in less than six days but only after more than 48 hours by ALS treatment (Bach & Dardenne, 1973). In contrast, background TRFC to SBC which are found in lymph nodes and peripheral blood are only moderately sensitive to azothioprine (Bach & Dardenne, 1973) and have both light and heavy chains on their surface (Greaves, quoted by Cantor, 1972c). They are unaffected by adult thymectomy but disappear after 6 hours of ALS treatment in vivo (Bach & Dardenne, 1973). After immunisation the spleen TRFC resemble background lymph node TRFC, being less sensitive to anti-theta serum and azothioprine (Bach & Dardenne, 1973).

Therefore it appears possible to distinguish two types of T cell. There is a population found predominantly in the spleen and thymus which is sensitive to adult thymectomy and thus dependent on the thymus, but insensitive to ALS and therefore sessile. After immunisation these cells appear to resemble those found in the lymph nodes and blood, that is, not dependent on the adult thymus, therefore possibly long-lived, but sensitive to AIS, and therefore circulating

cells. These two cell types have been termed T1 and T2 (Cantor, 1972c). Cantor further postulated (1972a, 1972b) that T1 and T2 belong to the same cell lineage, that T2 is a more mature form of T1 and that T1 is driven to T2 by antigen. He suggested that T2 cells are responsible for the T lymphocyte component of both the primary and secondary responses and that the only response of T1 is to become T2. The relation between T1 and T2 is purely speculative, but there is evidence to suggest that when thymus cells are injected into either syngeneic recipients with antigen (Miller & Mitchell, 1969) or into semi-allogeneic recipients (Sprent & Miller, 1972b; 1972c; 1971) they proliferate and after 4-5 days are able to circulate. There is further evidence to suggest a dissociation of T cell behaviour in cell mediated responses. It was shown that different cell populations had differing abilities to produce graft vs host responses, depending on whether it was measured by the mixed lymphocyte response, splenomegally or mortality assays. Thus it is conceivable that different T cell subpopulations are the main effectors in these assays (Tigelaar & Asofsky, 1972; Morse et al., 1974).

(b) Evidence for the existence of suppressor T cells

The evidence for the existence of suppressor T cells is not conclusive, especially where the target is postulated as another T cell population. There is however accumulating impressive evidence for the existence of a T cell population that suppresses antibody formation. By inference the possibility of T-T interactions cannot be excluded.

Experiments have suggested the possibility of feedback inhibition by T cells on certain types of antibody production such as the production of antibody to T independent antigens and homocytotropic antibody.

Animals which were depleted of T cells by either neonatal thymectomy or treatment with anti-lymphocytic sera or a combination of both produced an enhanced antibody response to keyhole limpet haemocyanin (Humphrey *et al.*, 1964; Baum *et al.*, 1969; Kerbal & Eidinger, 1971), PVP (Kerbal & Eidinger, 1971) and pneumococcal polysaccharide SIII (Humphrey *et al.*, 1964; Baker *et al.*, 1970a). In the majority of these responses an increase in  $\gamma M$  was observed but in some instances  $\gamma G$  increased (Kerbal & Eidinger, 1971; Baum *et al.*, 1969). It is therefore unlikely that thymectomy and treatment with anti-lymphocytic sera produced their effects by removing the  $\gamma G$  that would normally inhibit  $\gamma M$  (Uhr & Möller, 1968). Evidence that  $\gamma E$  production may be regulated by suppressor cells arises from experiments which showed that passive cutaneous anaphylaxis to dinitrophenyl (DNP)-Ascaris was enhanced in magnitude and duration in splenectomised and thymectomised rats (Okumura & Tada, 1971a). Moreover, the enhancement was prevented by the transfer of thymocytes from hyperimmune donors. Suppression by thymocytes was antigen specific and found not to be due to passive antibody. Haemagglutination titres of both total and ME-resistant antibody were normal.

Adoptive transfer of thymocytes has been shown to suppress, non-specifically, various antibody responses. For example, lethally irradiated mice were allowed to respond to horse red cells after they had been reconstituted with bone-marrow and normal spleen cells. The

addition however of cortisone resistant thymocytes or primed thymocytes to the reconstituting cell inocula inhibited restoration of the response (Eidinger & Pross, 1972). Similarly, the injection of thymocytes into irradiated recipients (700R) which had been reconstituted with bone-marrow did not elevate their response to Escherichia coli antigen and in some instances the  $\gamma$ M response was depressed (Möller & Michael, 1971). Thymocytes suppressed the ability of bone-marrow cells to produce antigen reactive cells to polymerised flagellin (Möller & Michael, 1971b). The existence of suppressor cells in the spleen was suggested by the findings that spleen cells which had been activated by concanavalin A when added with sheep erythrocytes to spleen cells in vitro, suppressed the  $\gamma$ M and  $\gamma$ G response to the antigen (Rich & Pierce, 1973).

Some types of immunological unresponsiveness have been explained by the presence of inhibitory T cells. Suggestions that T cells which have been intensely stimulated by antigen can become inhibitory and non-specifically suppress the ability of standby cells to respond to a different antigen within 1-10 days of the first would account for some instances of 'antigenic competition' (Adler, 1964; Radovich & Talmage, 1967). For example, mice which had been depleted of their T cells by neonatal thymectomy and irradiation, gave a normal response to horse erythrocytes four days after they had been injected with sheep erythrocytes (a procedure that in intact mice would abrogate the response to horse erythrocytes). Moreover, the simultaneous administration of  $6 \times 10^7$  thymocytes with the sheep erythrocytes inhibited the antibody response to horse red cells, and  $6 \times 10^7$

thymocytes restored antigenic competition more efficiently than  $1.5 \times 10^7$  thymocytes (Gershon & Kondo, 1971a). A similar phenomenon was reported to occur in vitro (Sjöberg & Britton, 1972). Sjöberg and Britton (1972) also demonstrated that cells from mice which had been immunised with Escherichia coli (a T independent antigen) could not produce antigenic competition although the response to Escherichia coli could be suppressed by cells from mice which had been immunised with sheep erythrocytes. This corroborates the suggestion that an antigen, in order to be able to initiate antigenic competition, needs to stimulate T cells.

Convincing evidence that B cells can be specifically inhibited from producing antibody arises from the phenomenon of 'allotypic suppression'. Mice (SJL X Balb/c) which had been bred from female Balb/c that had been immunised against the paternal allotype antibody (IgIb) did not produce IgIb and it was suggested that its production is actively suppressed (Jacobson & Herzenberg, 1972; Jacobson et al., 1972). Cells from these mice were found to suppress IgIb production in semi-lethally irradiated mice, and the suppressor cell found in the spleen, thymus, lymph node and bone-marrow was found to be a T cell (Herzenberg et al., 1973).

It has been suggested that in certain instances tolerance is actively maintained by thymocytes or peripheral T cells. Gershon & Kondo (1970) showed that the presence of thymocytes was essential to maintain the tolerant state of T dependent B cells. Spleens from mice which had been irradiated, reconstituted with bone-marrow cells and given a



tolerising regime of sheep erythrocytes and thymocytes were found able to transfer adoptive tolerance specifically to sheep cells, but only when thymocytes were used in the tolerising regime (Gershon & Kondo, 1971b). The active nature of the suppression was strengthened by the evidence of McCullagh (1970) who showed that tolerance in rats could not be broken by the injection of syngeneic thoracic duct cells.

Adoptive transfer of tolerance has also been achieved using peripheral lymphoid tissue. Low zone tolerance to bovine serum albumin (BSA) was transferred by spleen cells into normal mice (Terman et al., 1972). Inhibitory antibody produced by tolerant cells was implicated in the specific tolerant state which was produced by the transfer of lymph node cells from mice unresponsive to picryl chloride to normal mice (Asherson et al., 1971). Similarly spleen cells from mice which had been made tolerant to BSA, when injected into normal mice transferred specific tolerance (Crowle & Hu, 1969). Both of these responses measured delayed hypersensitivity. Lymph node cells can normally restore the response to picryl chloride when injected into irradiated mice which have been previously made unresponsive to picryl chloride, but when mixed with tolerant lymph node cells they were unable to do so (Asherson et al., 1971).

Allison et al. (1971) suggested that suppressor T cells may play an important role in regulating the immune response and preventing the onset of autoimmune disease. They suggested that T cells could perform this function by two mechanisms:

- (i) By being unresponsive to self-antigens, and thus not cooperating with B cells that have receptors for self-antigens.
- (ii) By exerting a specific feedback control on autoantibody formation.

Evidence for the second mechanism arises from experiments using NZB mice which normally develop symptoms of autoimmune diseases at about four months of age. Allison et al. (1971) suggest that this is due to a lack of suppressor T cells, for the transfer of young thymocytes to old mice prevented the onset of Coomb's positiveness, and this effect was abrogated by ALS treatment.

There is therefore a body of evidence accruing which suggests that T cells can suppress antibody formation. The mechanism of this suppression, whether by cell to cell contact or by production of some factor has not been elucidated. The importance of macrophages in the immune response and in particular the suggestion that in the absence of macrophages, or when the macrophage surface is saturated with IgT-antigen complexes, T cells become tolerogenic for B cells (Feldman, 1973) may contribute to the understanding of the mechanisms involved in T cell-mediated suppression.

The existence of a population of T cells that can inhibit antibody formation has to be reconciled with the knowledge that there is a population of T cells that help B cells produce antibody. The  $\gamma$ G response is sometimes more suppressed than the  $\delta$ M response, and this may suggest that the cooperative T cell is the target for suppression.

It has however been suggested that if B cells are directly suppressed by T cells then 'helper' function may simply represent suppression of the suppressor cells (Allison et al., 1971; Droege, 1973).

The evidence in favour of the existence of T cell sub-populations that synergise in graft vs host responses (Cantor, 1972a, 1972c) has been described. Experimental observations by R.K. Gershon and his colleagues suggest that there may be bidirectional effects of interaction between parental and  $F_1$  cells during a graft vs host response, producing synergy in some responses and suppression in others (Liebhaber et al., 1972; Gershon et al., 1972). Gershon suggests that the kinetics of the response by thymocytes to histocompatibility antigens implicates the presence of suppressor T cells (Gershon & Liebhaber, 1972). He postulates a regulatory role for these interactions, because high responder parental thymocytes are suppressed by the presence of  $F_1$  cells and low responder parental cells are boosted by  $F_1$  cells (Gershon et al., 1974). One of the difficulties of interpreting these results is that figures tend to be very variable, and differences are often small. This does not mean that the differences are not significant, but any definite opinion must await the advent of more data.

## MATERIALS AND METHODS

### Mice

Three to four month old male mice of the inbred strain CBA/H were used in the majority of experiments. Six to ten week old mice were used as thymocyte donors.

### Irradiation

Mice were placed in polystyrene boxes and exposed to lethal x-irradiation (900R) from a Westinghouse machine under the following conditions; 230kV; 15mA; 0.5mm Cu and 1.0mm Al filtration; dose-rate 66R/minute.

### Preparations of cell suspensions

Thymuses and lymph nodes were gently homogenised in ground glass tubes and the homogenate filtered through steel mesh. All cell suspensions were prepared in Hanks Balanced Salt Solution (Hanks BSS, Oxoid) pH 7.4 with sodium bicarbonate and kept at 0-4°C. Suspensions were washed three times and their viability assessed using Nigrosin (B.D.H.) immediately prior to the cells being injected into the tail vein in 0.4 mls of Hanks BSS.

### Peripheral blood lymphocytes

Mice were bled from the retroorbital plexus into heparinised tubes. The blood was washed three times in Hanks BSS and the volume adjusted to the required lymphocyte concentration in 0.4 mls. In some experiments the erythrocytes were removed with Plasmagel (Laboratoire Roger Bellon, Neuilly). 5 mls of blood were mixed with 2 mls of Plasmagel,

mixed and tilted to an angle of about  $45^{\circ}\text{C}$  and incubated for an hour at  $37^{\circ}\text{C}$ . During this period the tube containing the mixture was slowly turned upright. The red cells sedimented and the supernatant containing white cells was removed. In experiment 5, where the results are shown in table II.3, the blood was injected in 0.4 ml doses without prior treatment.

#### Cortisone treatment

The treatment recommended by Blomgren and Andersson (1969) was used. 2.5 mg of Hydrocortisone Acetate (Boots, Nottingham, England) were injected intraperitoneally into six to ten week old mice and they were killed two days later.

#### Anti-theta treatment

##### (a) Preparation of anti-theta serum

Anti-theta serum was raised in AKR mice by the method of Reif and Allen (1966). Three to four month old female AKR mice were given four weekly injections of  $10^7$  CBA thymus cells with  $2 \times 10^7$  pertussis organisms in 0.2 mls of saline intraperitoneally. Ten days after the last injection the mice were bled from the retroorbital plexus and the blood was allowed to clot. The serum was removed and non-specific cytotoxicity was absorbed by CBA spleen cells.

##### (b) Testing of anti-theta serum

0.1 ml of anti-theta serum, of various dilutions from 1:2 to 1:256 was added to 0.05 mls of rabbit complement and 0.1 ml of Hanks ESS containing  $10^6$  nucleated cells from bone-marrow, thymus, spleen or

lymph nodes. The cells and anti-theta/complement mixture were mixed and incubated for 45 minutes at 37°C. Viability was assessed using nigrosin. The lowest dilution found to give optimum killing of thymocytes was chosen to be used routinely.

c) Use of anti-theta serum

Anti-theta serum was added to 1 ml of Hanks BSS containing  $5 \times 10^6$  lymphocytes to make a final concentration of 1:8. They were incubated for 40 minutes at 37°C with occasional shaking. Guinea-pig or rabbit complement which had been freshly absorbed with mouse red blood cells was added to make a final concentration of 1:5. and incubation was continued for a further 20 minutes. The cells were washed three times in Hanks BSS and viability was assessed with nigrosin. Control suspensions were incubated under the same conditions using normal AKR serum and complement.

Thymectomies

Thymectomies were performed on mice when they were four weeks old. The technique was demonstrated to me by Helen Pritchard. The mice were examined post-mortem for the presence of thymic remnants.

Antigens

a) Oxazolone

2-ethoxy-methylene-4-phenyl-oxazolone (British Drug Houses) was dissolved in absolute alcohol at 60°C at 10% w/v. Two methods of application were used:

- (i) 0.05mls (5mg) was applied using a pipette to a shaved area approximately 2 x 1 cm on the left side of the thorax. Control mice were shaved and received hot ethanol only.
- (ii) 0.05mls (5mg) was applied to the left hind foot pad of an anaesthetised (Nembutal BDH) mouse and the foot was immobilised by binding it with Plaster of Paris Bandage ("Gypsona", T.J. Smith and Nephew Ltd., England). Control mice were anaesthetised and given hot alcohol and their feet bound with Gypsona bandage.

b) Ferritin

0.1mg of Ferritin (Koch-Light Labs., Bucks) in 0.05 mls of saline was injected subcutaneously into right foot-pads. Control mice received saline only.

c) Polyvinylpyrrolidone (PVP)

PVP, m.wt. 340,000 was kindly provided by Henry Clamen. 2 µgm were injected in 0.05 ml of saline subcutaneously into the right fore-foot pads.

Measurement of DNA synthesis

DNA synthesis was measured by incorporation of  $^{125}\text{I}$ -labelled 5-iodo-2'-deoxyuridine ( $^{125}\text{I}$ -UdR) (Radiochemical Centre, Amersham) after the method of Hughes et al. (1964) modified by Pritchard and Micklem (1972). Mice received  $5 \times 10^{-8}$  moles of FUDR (Roche Products Ltd.) in 0.2 mls of saline intra-peritoneally followed one hour later by 1 µCi of  $^{125}\text{I}$ -UdR, adjusted to a specific activity of 5 µCi/mg, by the same route.

Mice were killed two hours later, their lymph nodes excised and fixed in formal-ethanol (4% formalin in 70% ethanol). Unincorporated label was removed by several changes of 70% ethanol over a period of 5-7 days, until the washings contained no more than twice background counts (Elkins, 1970).

Axillary and brachial nodes from one side of the animal were counted together in an Auto-Packard Gamma counter. The injected dose was also counted. The organs were counted for 5 minutes, background subtracted, and the counts per minute were adjusted to the counts that would have been obtained from an injected dose of  $10^6$  cpm. The calculation was as follows:

$$^{125}\text{I-UdR uptake} = \log_{10} \left[ \frac{\text{counts in organ (cpm)} \times 10^6}{\text{injected dose (cpm)}} \right]$$

It can be seen that the terms 'cpm' cancel out, and the  $^{125}\text{I-UdR uptake}$  is therefore the  $\log_{10}$  of the proportion of the injected dose that was incorporated.

### Statistical analysis

The statistical significance of differences between results was assessed using the Student's T Test.



## RESULTS

### 1. The response of normal mice to different doses of oxazolone

These responses were measured 3 days after the administration of the antigen.

#### a) The effect of painting different doses of oxazolone on the flank. Fig. II.1; Table II.1.

The results recorded in Fig. II.1 show that the lymph nodes which received the greatest stimulation, measured by the uptake of  $^{125}\text{I}$ -UdR, were the regional axillary and brachial nodes. A range of oxazolone doses from 0.1 mg to 10 mg produced no differences in the amount of  $^{125}\text{I}$ -UdR incorporated by the draining nodes. Doses of antigen above 3 mg did however stimulate the contralateral axillary and brachial nodes and increased the amount of  $^{125}\text{I}$ -UdR incorporated by the inguinal and popliteal lymph nodes. There are no data reporting the effect of alcohol on DNA synthesis within the inguinal and popliteal nodes. It is notable that the administration of oxazolone on the left flank stimulated DNA synthesis by the spleen. Table II.1 shows that increasing the dose of oxazolone, especially between 1-10 mg decreased the ratio of  $^{125}\text{I}$ -UdR which was incorporated in the draining/contralateral axillary and brachial lymph nodes. It did not however decrease the ratio of label which was incorporated in the draining/contralateral inguinal and popliteal nodes, where the ratio was consistently unity.

#### b) The effect of painting different doses of oxazolone on the hind foot pad. Fig. II.2; Table II.2.

The results shown in Fig. II.2 demonstrate that the lymph nodes which

received the greatest amount of stimulation by oxazolone were those nearest the point of application, the inguinal and popliteal lymph nodes. In contrast, in the axillary and brachial nodes there was no indication of a proliferative response specific to oxazolone below 1 mg of antigen and possibly there was no response to doses of 1-10 mg. There was no dose dependent increase in the amount of proliferation found in any of the lymph nodes, and Table II.2 shows that there was no dose-dependent change in the ratio of  $^{125}\text{I}$ -UdR uptake between the draining and contralateral nodes. The variability of these results is noticeable compared to the results reported in Table II.1. The spleen appears to remain hardly stimulated when the foot-pad is painted. The results in Table II.2 show that the inguinal nodes gave consistently more proliferation in the draining than the contralateral nodes, down to 0.3 mg. of oxazolone. The ratio of the amount of  $^{125}\text{I}$ -UdR incorporated between the draining and contralateral axillary nodes was unity except for the dose of 1 mg where the ratio was  $3.04 \pm 1.67$ .

## 2. Timing of the experimental procedures. Fig. II.3

The experiments which were to be utilised in this series of studies (Chapter I) involved lethally irradiating (900R) mice, repopulating them with different doses of various cell suspensions, and sensitising them with 5 mg of oxazolone. Pilot studies (A.C. Payne, pers. comm.) in this laboratory indicated that there could be an advantage to delaying the application of oxazolone until one day post irradiation and repopulation. This suggestion was formally tested. Three groups of mice were subjected to lethal irradiation, were injected with three doses of thymocytes,  $10^6$ ,  $5 \times 10^6$  and  $5 \times 10^7$  and were painted with oxazolone according to

the following schedule:

- Group A: were irradiated, three hours later they were repopulated and 24 hours after repopulation they were painted with oxazolone.
- Group B: were irradiated, three hours later they were repopulated and three hours after repopulation they were given oxazolone.
- Group C: were irradiated, three hours later they were painted with oxazolone and three hours after antigen they were injected with thymocytes.

All responses were measured by estimating the amount of  $^{125}\text{I}$ -UdR incorporated by the draining lymph nodes 4 days after the application of oxazolone. The results in Fig. II.3 show that lymph nodes from animals in Group A produced significantly higher proliferative responses than those from Group B, at all thymocyte doses. There were no differences between the responses of nodes from mice in Groups A and B to alcohol. Animals from Group A also incorporated more  $^{125}\text{I}$ -UdR in their draining lymph nodes than did mice from Group C, when injected with  $10^6$  and  $5 \times 10^7$  thymocytes.

3. Cell transfer experiments demonstrating the response of lymphocytes from different anatomical regions to oxazolone

In these experiments, mice were lethally irradiated (900R), repopulated on the same day and sensitised with 5 mg of oxazolone on the left flank one day later. DNA synthesis was measured in the axillary and brachial lymph nodes, and spleen, 4 days or 3, 4 and 5 days after oxazolone.



a) Thymocytes(i) The response of low doses of thymocytes in lymph nodes of irradiated mice: Fig. II.4.

The proliferative response of  $3.6 \times 10^5$ ,  $1.2 \times 10^6$  and  $3.6 \times 10^6$  thymocytes were measured 4 days after oxazolone. The amount of  $^{125}\text{I-UdR}$  incorporated in the lymph nodes of the irradiated recipients increased concomitantly with the number of thymocytes which had been given. The smallest number of thymocytes ( $3.6 \times 10^5$ ) were sufficient to produce a response significantly greater than the controls response to alcohol (P 0.01-0.005). The various thymocyte inocula did not proliferate in response to alcohol to a significant degree.

(ii) The response of high doses of thymocytes in lymph nodes of irradiated mice: Fig. II.5.

Although this was a different experiment to that performed using low doses of thymocytes (as described above), the results are corrected to a standard injected dose and are therefore, to a certain extent, comparable between experiments. The proliferative response of  $5 \times 10^7$  cells to oxazolone was probably greater than that of  $3 \times 10^6$  cells (Fig. II.4), but thereafter increasing the number of thymocytes that were injected was quite ineffective in increasing the amount of  $^{125}\text{I-UdR}$  incorporated by lymph nodes. There are no groups that were given alcohol, and these results may overestimate the response to oxazolone because some of the incorporated isotope could be due to non-specific stimuli. The proliferative response of thymocytes to oxazolone thus plateaus at about  $5 \times 10^7$  cells.

(iii) The response of thymocytes in spleens of irradiated mice.  
Fig. II.6; Fig. II.7.

Spleens were obtained from the mice which were used in experiments (i) and (ii) described above.

There was no significant proliferation of  $3.6 \times 10^5$ ,  $1.2 \times 10^6$  or  $3 \times 10^6$  thymocytes in the spleens of irradiated recipients. There were no groups of animals which had received the higher doses of thymocytes and alcohol, and in the absence of this control it is not possible to conclude that thymocytes were responding to oxazolone. The level of  $^{125}\text{I}$ -UdR incorporation was below that found in lymph nodes.

(iv) The response of thymocytes 3, 4 and 5 days after the administration of oxazolone. Fig. II.8.

The results obtained from experiments (i) and (ii) suggested that  $5 \times 10^7$  thymocytes give a maximum response to oxazolone that cannot be superseded by higher numbers of thymocytes. It is conceivable however that a high dose of cells gives a maximum response at an earlier time than a low dose. Thus the conclusion drawn from experiments (i) and (ii) must be verified by measuring the response of different thymocyte doses 3, 4 and 5 days after oxazolone. The response of  $3 \times 10^5$ ,  $3 \times 10^6$ ,  $10^7$  and  $7 \times 10^7$  thymocytes was measured. Lymph nodes from animals which had been injected with  $10^7$  or  $3 \times 10^6$  showed no differences in the amount of  $^{125}\text{I}$ -UdR incorporated, on any of the days tested. On day 3, the response from  $3 \times 10^6$  thymocytes was significantly greater than that from  $3 \times 10^5$  cells. The response from  $7 \times 10^7$  cells was greater than all other doses. On day 4, the mice which had been injected with  $7 \times 10^7$  cells gave a similar response

to those that had been injected with  $10^7$  cells. Similarly, on day 5 those mice which had been inoculated with  $7 \times 10^7$  gave a similar response to those inoculated with  $10^7$  cells and there was no significant difference between the response of animals injected with  $3 \times 10^6$  thymocytes and  $7 \times 10^7$  thymocytes ( $P$  0.15-0.10). The response from animals which had been injected with the lowest number of thymocytes  $3 \times 10^5$  was consistently the lowest response.

These results demonstrated that:

(i) High numbers of thymocytes, when injected into irradiated recipients gave their maximum proliferative response to oxazolone earlier than low numbers of thymocytes.

(ii) There was no difference in the maximum amount of  $^{125}\text{I}$ -UdR incorporated by lymph nodes of animals which had been injected with  $7 \times 10^7$  thymocytes and those injected with  $3 \times 10^6$  thymocytes. Thus within a certain dose range, a twenty-fold increase in cell number does not produce any increase in proliferation.

(iii) The maximum amount of  $^{125}\text{I}$ -UdR incorporated by the lymph nodes of animals which had been injected with  $7 \times 10^7$  cells was significantly below the amount incorporated by unirradiated animals.

b) Cortisone resistant thymocytes. Fig. II.2

Thymuses were removed from mice 2 days after they had received 2.5 mg of cortisone acetate. They were much reduced in size and cell counts showed that they contained 5-10% the number of cells found in thymuses

from untreated mice.

The proliferative responses of lymph nodes from irradiated mice which had been injected with  $10^6$  normal and  $10^6$  cortisone resistant thymocytes were compared 3, 4 and 5 days after the administration of oxazolone. Mice which had been irradiated, injected with thymocytes and painted with alcohol were killed on day 4. The results in Fig. II.9 show that the lymph nodes from mice which had been injected with normal thymocytes produced a steady increase in the amount of  $^{125}\text{I}$ -UdR incorporated between days 3 and 5, in contrast to the lymph nodes from mice which had been injected with cortisone resistant thymocytes, these showed similar amounts of isotope incorporation on days 4 and 5. The amount of proliferation from cortisone resistant thymocytes was significantly greater on each day than that produced by normal thymocytes (day 3,  $P$  0.025-0.0125; day 4,  $P$  0.0025-0.0005; day 5,  $P$  0.0125). The differences in reactivity between the thymocyte populations is illustrated by the observation that on day 3 the response from normal thymocytes was hardly greater than the control value ( $P$  0.05) while the response from cortisone resistant thymocytes was significant ( $P$  0.05-0.025).

The following two experiments, (c) and (d) were done in collaboration with N. Anderson and H.S. Micklem.

c) Lymph node lymphocytes, Fig. II.10.

Lymphocytes which were obtained from the pooled axillary, brachial and inguinal nodes were injected into irradiated recipients in doses

ranging from  $10^3$  -  $8.8 \times 10^6$  cells/mouse. The minimum number which was found necessary to allow the lymph nodes of the recipients to produce a significant proliferative response to oxazolone, 4 days after its administration, was  $3 \times 10^4$ ; the response increased in magnitude concomitant with the cell increments, particularly within the dose range,  $10^5$  -  $10^6$ . The greatest response was given by mice which had been injected with the greatest number of lymphocytes, that is  $8.8 \times 10^6$  cells. The response produced by  $8.8 \times 10^6$  cells was significantly lower than that produced by unirradiated mice ( $P = 0.025 - 0.0125$ ).

d) Peripheral blood lymphocytes. Fig. II.11

Fig. II.11 incorporates the results from two experiments which involved measuring the incorporation of  $^{125}\text{I}$ -UdR by lymph nodes of irradiated mice which had been injected with peripheral blood lymphocytes.

In one experiment, blood lymphocytes were injected in doses ranging from  $5.3 \times 10^3$  -  $1.8 \times 10^6$  cells/mouse, and the response to oxazolone was measured 4 days after oxazolone. In the second experiment the number of cells which were injected varied from  $3.5 \times 10^5$  -  $8 \times 10^6$ /mouse and the blood was passed through plasmagel to remove red blood cells. The response of animals which had been injected with  $3.5 \times 10^5$  -  $8 \times 10^6$  cells was measured 4 days after oxazolone and the response of animals which had been injected with  $3 \times 10^5$  cells and  $10^6$  cells was assayed on day 5 as well as on day 4. The lowest number of lymphocytes which were found capable of transferring a response to oxazolone was  $5.3 \times 10^4$  cells. The magnitude of the response increased as the number of transferred cells was increased and the greatest increase



occurred within the range  $5.3 \times 10^4$  to  $3 \times 10^6$  cells. Thereafter the response possibly tended to plateau between  $3 \times 10^6$  and  $8 \times 10^6$  cells. The response of mice which had been injected with  $8 \times 10^6$  cells was significantly below the proliferative response of unirradiated mice, 4 days after oxazolone. Animals which had been injected with  $3 \times 10^5$  and  $10^6$  lymphocytes produced a considerable increase in their uptake of  $^{125}\text{I}$ -UdR between days 4 and 5. The amount of label incorporated by lymph nodes of mice which had been injected with  $10^6$  cells was within the range of response produced by unirradiated animals 5 days after oxazolone.

4. Experiment to investigate the effect of thymocytes and cortisone resistant thymocytes on the response of lymph node cells to oxazolone. Fig. II.12

Mice were irradiated and given injections of various lymphocyte types, or mixtures of lymphocyte types according to the following schedule;

No. and type of lymphocytes

<u>Group</u>	<u>Lymph node lymphocytes</u>	<u>Thymocytes</u>	<u>Cortisone resistant thymocytes</u>
A	$3 \times 10^5$	-	-
B	-	$10^6$	-
C	-	-	$10^5$
D	$3 \times 10^5$	$10^6$	-
E	$3 \times 10^5$	-	$10^5$

The above groups were duplicated and half were painted with oxazolone and half with alcohol. The animals were injected with  $^{125}\text{I}$ -UdR 4 days after the administration of oxazolone or alcohol and the amount of

isotope incorporated in their lymph nodes was measured. A dose of  $3 \times 10^5$  lymphocytes was chosen because, as can be seen from Fig. II.10, any change in the responsiveness of cells at this dose should result in a detectable enhancing or depressing effect. Animals which had been injected with  $3 \times 10^5$  lymphocytes gave a significant proliferative response to oxazolone. The responses produced from mice which had been injected with  $10^6$  thymocytes or  $10^5$  cortisone resistant thymocytes was not above that produced by the alcohol controls; this was unexpected. The magnitude of the response that was transferred by a mixture of  $3 \times 10^5$  lymph node lymphocytes and  $10^6$  thymocytes was equivalent to that transferred by  $3 \times 10^5$  lymphocytes alone. Similarly, mice which had been injected with a mixture of  $3 \times 10^5$  lymphocytes and  $10^5$  cortisone resistant thymocytes produced the same amount of  $^{125}\text{I}$ -UdR incorporation as did mice which had been injected with lymphocytes alone.

5. The proliferative response of lymphocytes from animals previously immunised with oxazolone

Animals were irradiated and given injections of lymphocytes from donors which had received either 10 mg of oxazolone or 0.1 ml of alcohol on shaved areas of both sides of the flank 14 days previously. In order to ascertain the T cell component of these lymphocyte populations, and whether they participate in the 'secondary' proliferative response to oxazolone, some mice were injected with cell suspensions which had been treated with anti-theta serum and rabbit complement. Cell suspensions which were not treated with anti-theta serum were incubated with normal mouse AKR serum and rabbit complement.

The anti-theta serum produced a cytotoxic index on normal lymphocytes of about 28% and on lymphocytes from immunised mice of about 66%. Irradiated recipients received injections of either  $3 \times 10^5$  normal lymphocytes,  $3 \times 10^5$  normal lymphocytes which had survived anti-theta treatment,  $3 \times 10^5$  lymphocytes from donors which had been immunised to oxazolone or  $3 \times 10^5$  lymphocytes from immunised donors after treatment with anti-theta serum. The mice received either oxazolone or alcohol one day after the cell suspensions, and were injected with  $^{125}\text{I-UdR}$  4 days later. The lymph nodes from mice which had been injected with cells from immunised donors, incorporated significantly more  $^{125}\text{I-UdR}$  ( $2.9 \pm 0.05$ ) than the lymph nodes from mice which had received normal cells ( $2.4 \pm 0.06$ ). The ability of normal lymphocytes to transfer the proliferative response was depressed after treatment with anti-theta serum, although the response was greater than that achieved by the alcohol controls. The elevation of the proliferative response produced in irradiated recipients by the transfer of cells from immunised donors was partially abrogated when the transferred cells were incubated with anti-theta serum, although it remained significantly above the response produced by a similarly treated group of animals which had received alcohol. It is interesting that the response was also significantly higher than the response transferred by anti-theta serum treated normal lymphocytes.

6. The effect of adult thymectomy on the ability of lymph node and peripheral blood lymphocytes to transfer the proliferative response to oxazolone. Table II.3

The results from two experiments are shown in Table II.3. Mice were thymectomised when they were 4 weeks old and were used to donate lymph

node and blood cells 6 months later in experiment I and eight months later in experiment II. The controls consisted of mice of the same age that had not been thymectomised and young (10 week old) mice.

In experiment I, irradiated recipients received injections of one of the following cell suspensions:

- (i) peripheral blood cells or lymph node cells from thymectomised donors.
- (ii) peripheral blood or lymph node cells from 7 month old donors.
- (iii) peripheral blood or lymph node cells from 10 week old donors.

The mice were painted with oxazolone or alcohol the following day and injected with  $^{125}\text{I}$ -UdR 3 and 4 days after the administration of the antigen. Post-mortem examination of thymectomised donors revealed the presence of one thymus lobe in one of the eight thymectomised donors. The lymph nodes from this mouse were not used in the experiment but the blood unfortunately was. The results show that recipients of  $2.18 \times 10^6$  peripheral blood lymphocytes from thymectomised donors gave a response on day 3 that was, cell for cell, significantly below that produced by recipients of  $2.72 \times 10^6$  peripheral blood lymphocytes from normal donors of the same age. Comparison of the response of the recipients of cells from thymectomised donors with that of the recipients of peripheral blood lymphocytes from young mice which were painted with alcohol demonstrates that peripheral blood lymphocytes failed to transfer a response to oxazolone, on day 3. On day 4 however, the recipients of cells from thymectomised mice produced a response to oxazolone significantly above the alcohol control ( $P\ 0.05 - 0.025$ ). The response would not appear to be inferior to that produced by

peripheral blood lymphocytes from normal 7 month old donors. Similarly, mice which had received lymph node cells from thymectomised or normal 7-month old donors showed little difference in their ability to respond to oxazolone, 4 days after oxazolone. A comparison of the abilities of lymph node cells from normal and thymectomised donors to transfer the 3 day response to oxazolone cannot be made because the results pertaining to the response of lymph node cells from normal 7-month old donors are not available.

There was no significant difference between the responsiveness of lymph node cells from young mice and from 7-month old mice (P 0.01).

In experiment II mice were irradiated and given injections of one of the following:

- (i)  $0.5 \times 10^6$  lymph node lymphocytes from thymectomised donors.
- (ii)  $0.5 \times 10^6$  lymph node cells from normal donors of the same age as the thymectomised donors.
- (iii)  $0.5 \times 10^6$  lymph node cells from 10 week old donors.

The mice were painted with oxazolone or alcohol the following day and injected with  $^{125}\text{I}$ -UdR 4 days after administration of the antigen.

Post-mortem examination of the thymectomised donors revealed no traces of thymus. The results demonstrate that there was no difference in the ability of lymph node lymphocytes from 9 month old thymectomised and 9 month old normal donors to transfer a 4 day proliferative response to oxazolone ( $2.5 \pm 0.038$  compared to  $2.47 \pm 0.05$ ). The responses which were transferred were significantly higher than those transferred by the

same number of lymphocytes from 10 weekold mice ( $P\ 0.025$ ). If the response of the cells from the young mice ( $2.33 \pm 0.05$ ) is compared to the lymph node dose response curve in Fig. II.10 (compiled using 3 month old mice), it can be seen that the response is below that which would be predicted from Fig. II.10, that is a value of about 2.6. Thus, possibly the young mice which were used in experiment II were producing an abnormally low response.

7. The proliferative response of thymocytes to ferritin and PVP. Figs. II.14 and II.15.

Irradiated recipients received injections of  $10^6$ ,  $10^7$ ,  $3 \times 10^7$  and  $10^8$  thymocytes and on the same day they were injected in the left fore foot pad with either ferritin or PVP. Control groups of irradiated mice received the same schedule of injections of thymocytes and were given saline subcutaneously in the fore foot pad.  $^{125}\text{I}$ -UdR was injected 3, 4 or 5 days later and the incorporation in the draining lymph nodes was estimated. The incorporation of  $^{125}\text{I}$ -UdR in response to ferritin is shown in Fig. II.14. Mice which had been injected with either  $10^6$  or  $10^7$  thymocytes failed to produce a DNA synthetic response on days 3, 4 or 5. On day 5, although the response of mice which had received high doses of thymocytes, that is  $10^8$  and  $3 \times 10^7$  thymocytes was significantly above the response of the saline controls, the response of  $3 \times 10^7$  cells ( $2.24 \pm 0.08$ ) was only equivalent to that of  $3 \times 10^5$  thymocytes responding to oxazolone (Fig. II.8).

The proliferation of thymocytes in response to stimulation by PVP is shown in Fig. II.15. Mice which had been injected with  $3 \times 10^7$  cells

failed to respond on days 3, 4 or 5. mice which had been injected with  $10^8$  thymocytes produced a proliferative response on day 5 of  $2.26 \pm 0.14$ , a value significantly above the saline control which had received  $10^8$  thymocytes (P 0.01). The results on day 4 are difficult to interpret due to the high levels of proliferation achieved by animals which received injections of saline. These results are notably variable compared with the results obtained using oxazolone.

Fig. II.1

<sup>125</sup>I-UdR incorporation by various lymph nodes and the spleen of mice, 3 days after receiving different doses of oxazolone or alcohol administered on the left flank.

Recipients of oxazolone; incorporation by the draining ■ and contralateral □ axillary and brachial lymph nodes; draining ▲ and contralateral △ inguinal nodes; draining ● and contralateral ○ popliteal nodes; ◆ spleen.

Recipients of alcohol; incorporation by the axillary and brachial nodes ▼ (shaded area) and spleen ◇ (shaded area).



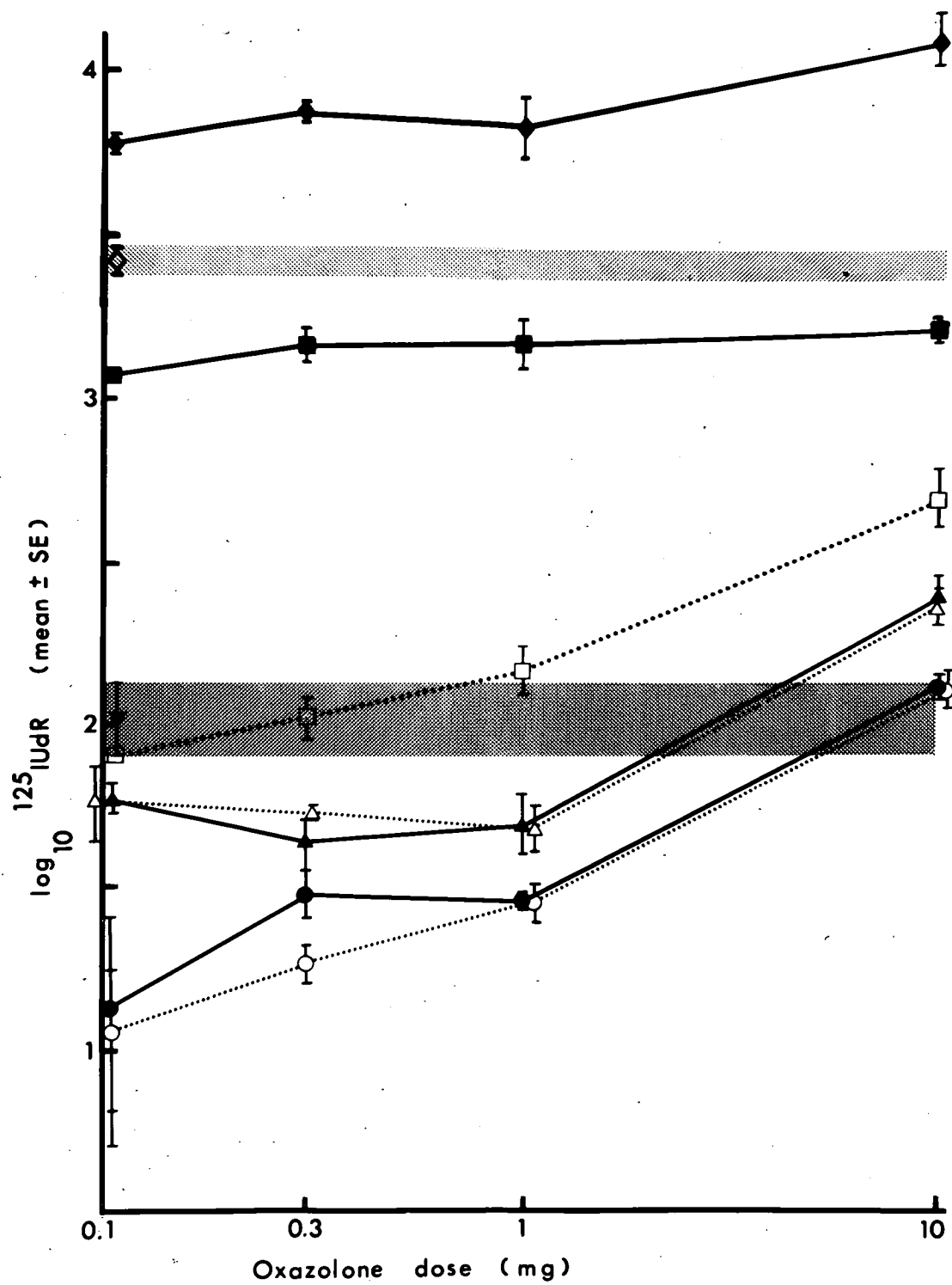


Fig. 11.2

<sup>126</sup>I-UdR incorporation by various lymph nodes and the spleen of mice, 3 days after receiving different doses of oxazolone or alcohol, administered on the left hind foot pad.

Recipients of oxazolone; incorporation by the draining ■ and contralateral

□ inguinal nodes; draining ▲ and contralateral △ axillary and brachial nodes; draining ◆ and contralateral ◇ popliteal nodes; ● spleen.

Recipients of alcohol; incorporation by the draining ▼ and contralateral

▽ inguinal nodes; draining ◆ and contralateral ⊕ axillary and brachial nodes; draining ▼ and contralateral △ popliteal nodes;

○ spleen (shaded area).

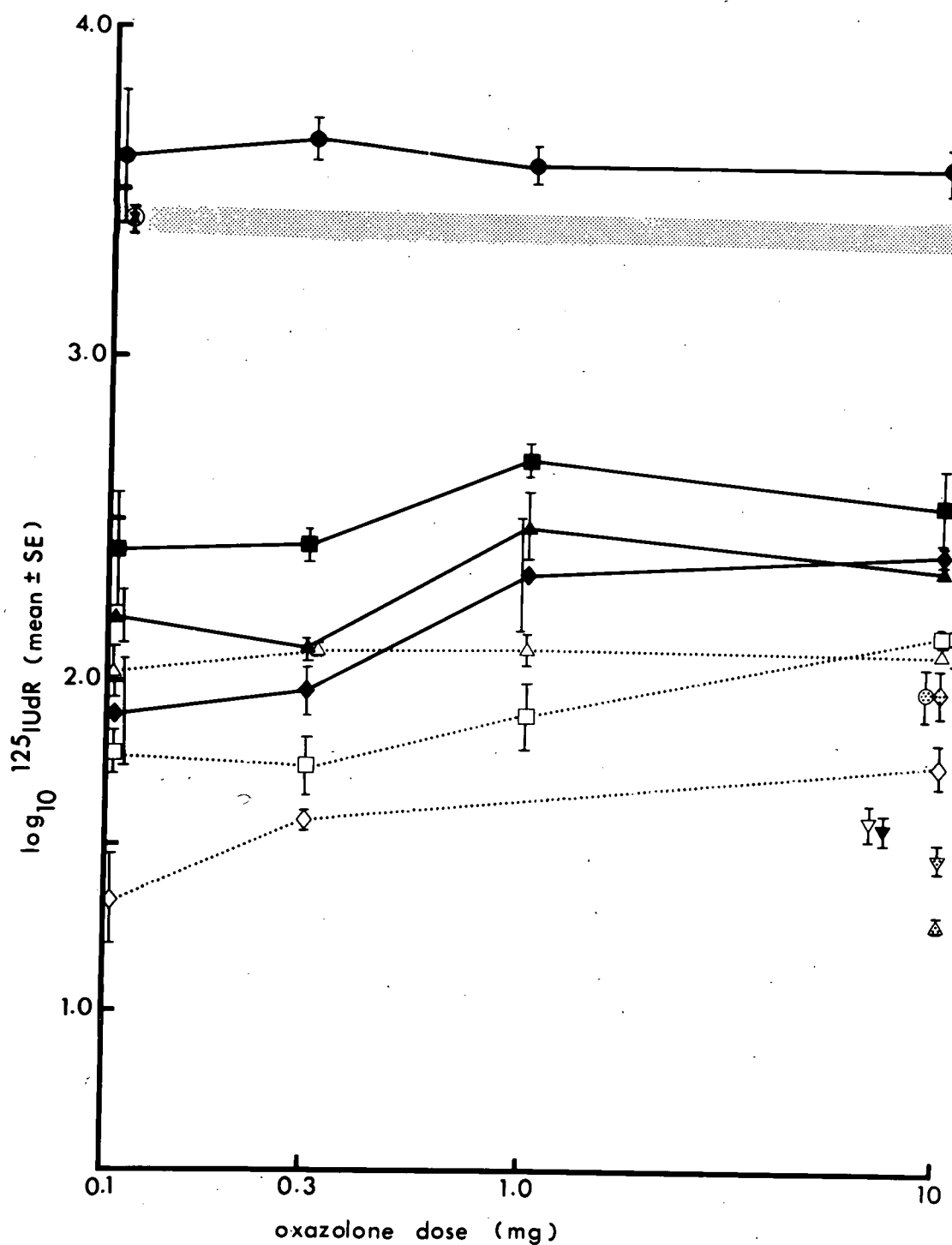


Fig. 11.3

<sup>125</sup>I-UdR incorporation by the axillary and brachial lymph nodes of irradiated recipients of thymocytes in response to oxazolone which was administered at different times relative to irradiation and thymocyte-repopulation.

Group A ▲ ; group B ■ ; group C ● . Mice which were irradiated and received oxazolone as in A, but received saline instead of thymocytes Δ ; mice which were irradiated and received oxazolone as in B, but received saline instead of thymocytes □ .

Explanation of the groups, A, B and C is found in the text.

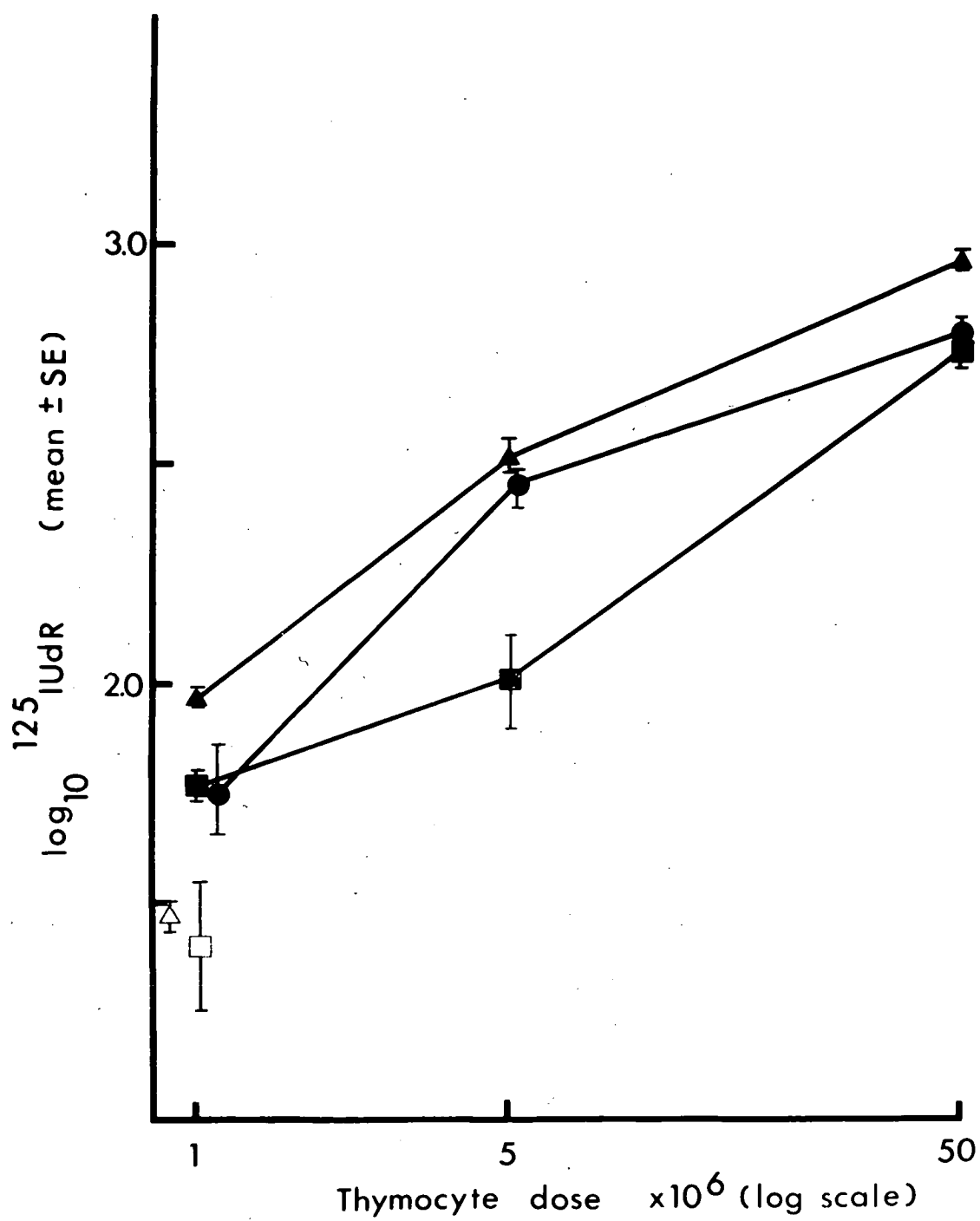


Fig. II.4

<sup>125</sup>I-UdR incorporation by the lymph nodes of irradiated recipients of 'low' doses of thymocytes, 4 days after receiving oxazolone or alcohol.

The response in irradiated recipients of thymocytes and oxazolone ▲ , of thymocytes and alcohol △ , of saline and oxazolone ● , of saline and alcohol ○ ; in normal recipients of oxazolone ■ or alcohol □ .

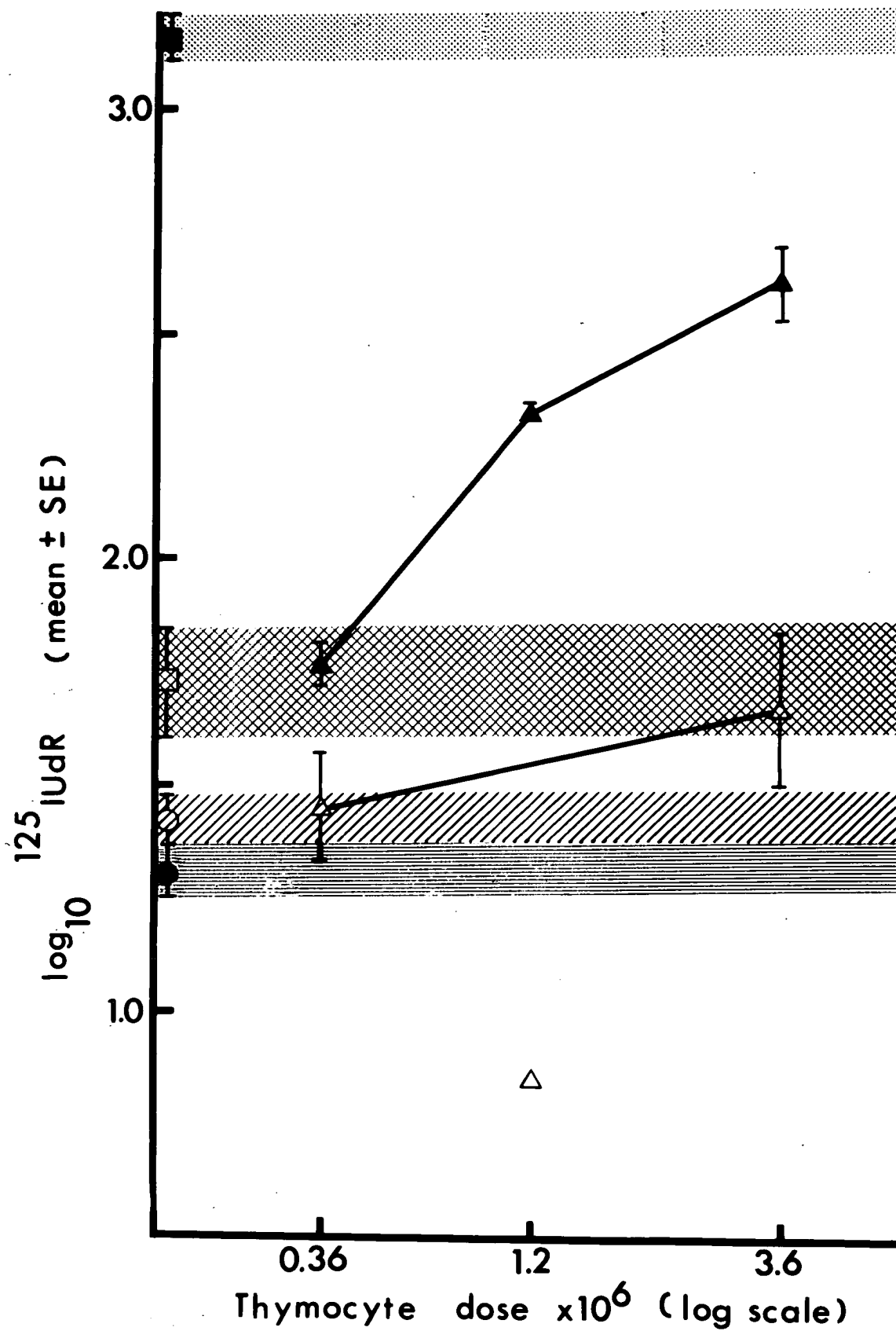


Fig. II.6

$^{125}$ I-UdR incorporation by the lymph nodes of irradiated recipients of 'high' doses of thymocytes, 4 days after receiving oxazolone.

The response in irradiated recipients of thymocytes and oxazolone ▲ , of saline and oxazolone ● , of saline and alcohol ○ ; in normal recipients of oxazolone ■ or alcohol □ .



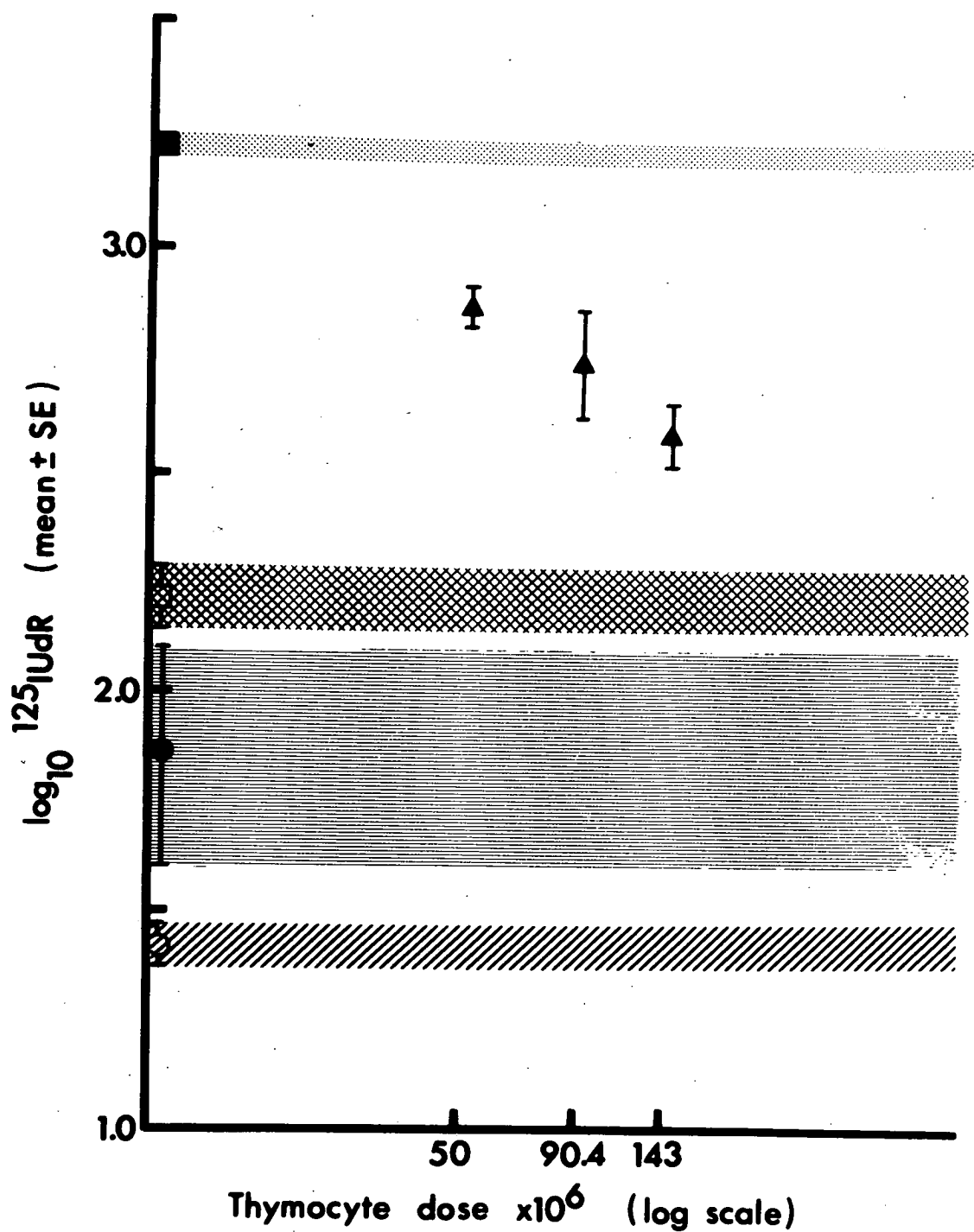


Fig. 11.6

<sup>125</sup>IUdR incorporation by the spleens of irradiated recipients of 'low' doses of thymocytes, 4 days after receiving oxazolone or alcohol. The response in irradiated recipients of thymocytes and oxazolone ▲ , of thymocytes and alcohol △ , of saline and oxazolone ● , of saline and alcohol ○ ; in normal recipients of oxazolone ■ or alcohol □ .

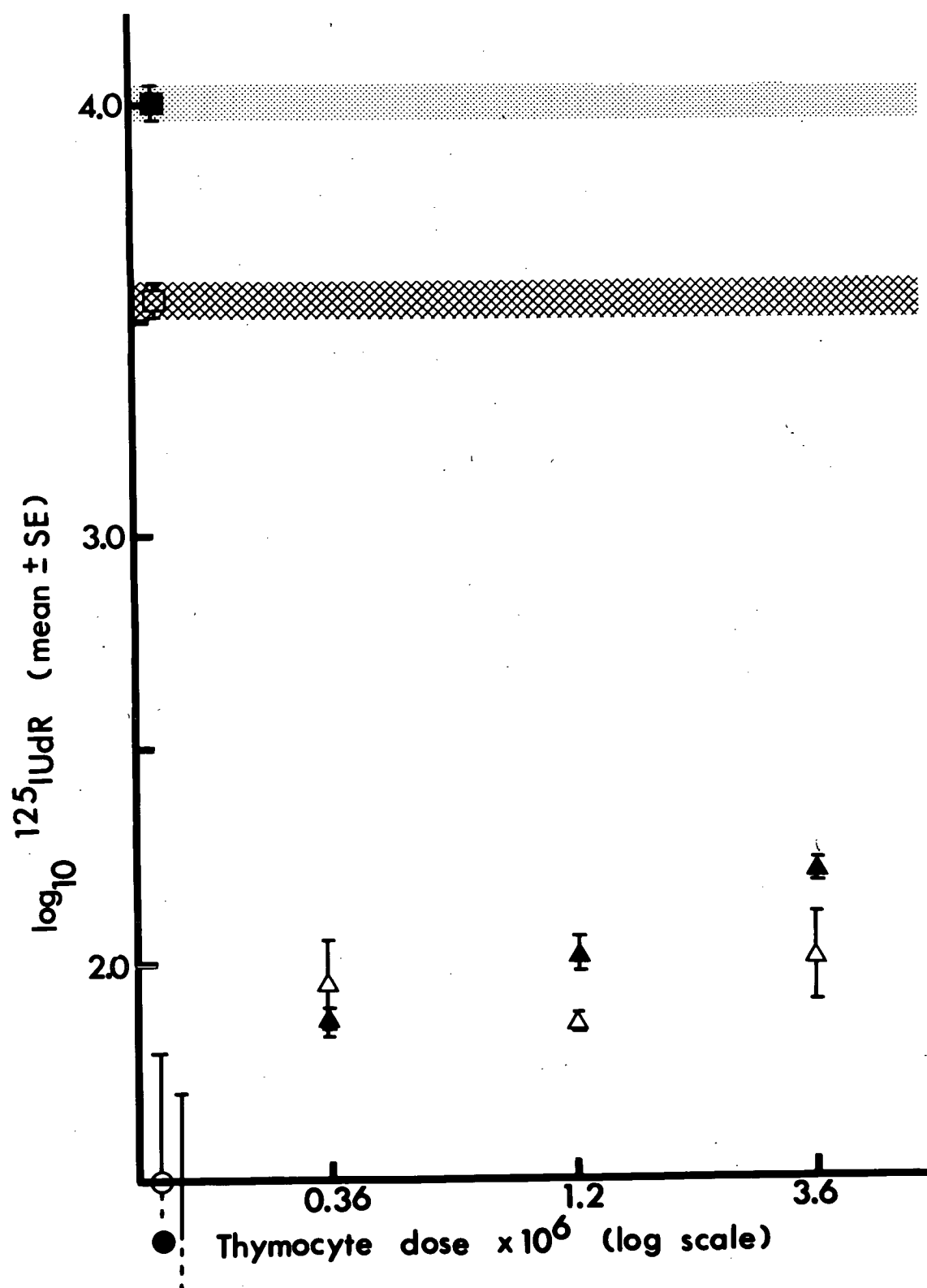


Fig. II.7

<sup>125</sup>IUdR incorporation by the spleens of irradiated recipients of 'high' doses of thymocytes, 4 days after receiving oxazolone or alcohol.

The response in irradiated recipients of thymocytes and oxazolone ▲ , of saline and oxazolone ◆ , of saline and alcohol △ ; in normal recipients of oxazolone ■ or alcohol □ .

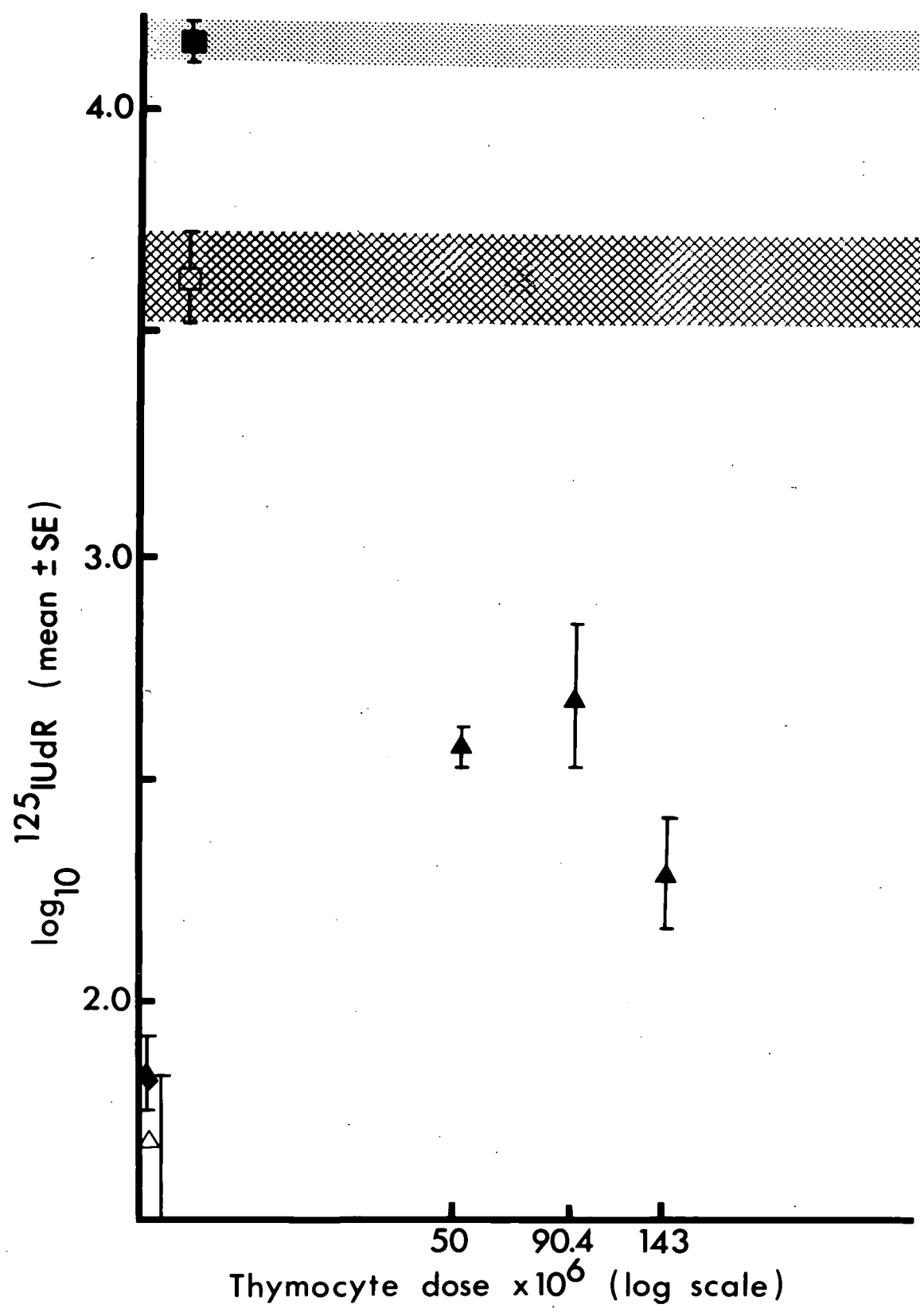


Fig. II.8

$^{125}$ IUdR incorporation by the lymph nodes of irradiated recipients of different doses of thymocytes, 3, 4 and 6 days after receiving oxazolone.

The response in irradiated recipients of oxazolone and  $3 \times 10^5$  thymocytes  $\blacktriangle$ , oxazolone and  $3 \times 10^6$  thymocytes  $\blacktriangledown$ , oxazolone and  $10^7$  thymocytes  $\blacklozenge$ , oxazolone and  $7 \times 10^7$  thymocytes  $\blacklozenge$ , of oxazolone and saline  $\bullet$ , of alcohol and saline  $\circ$ ; in normal recipients of oxazolone  $\blacksquare$ .

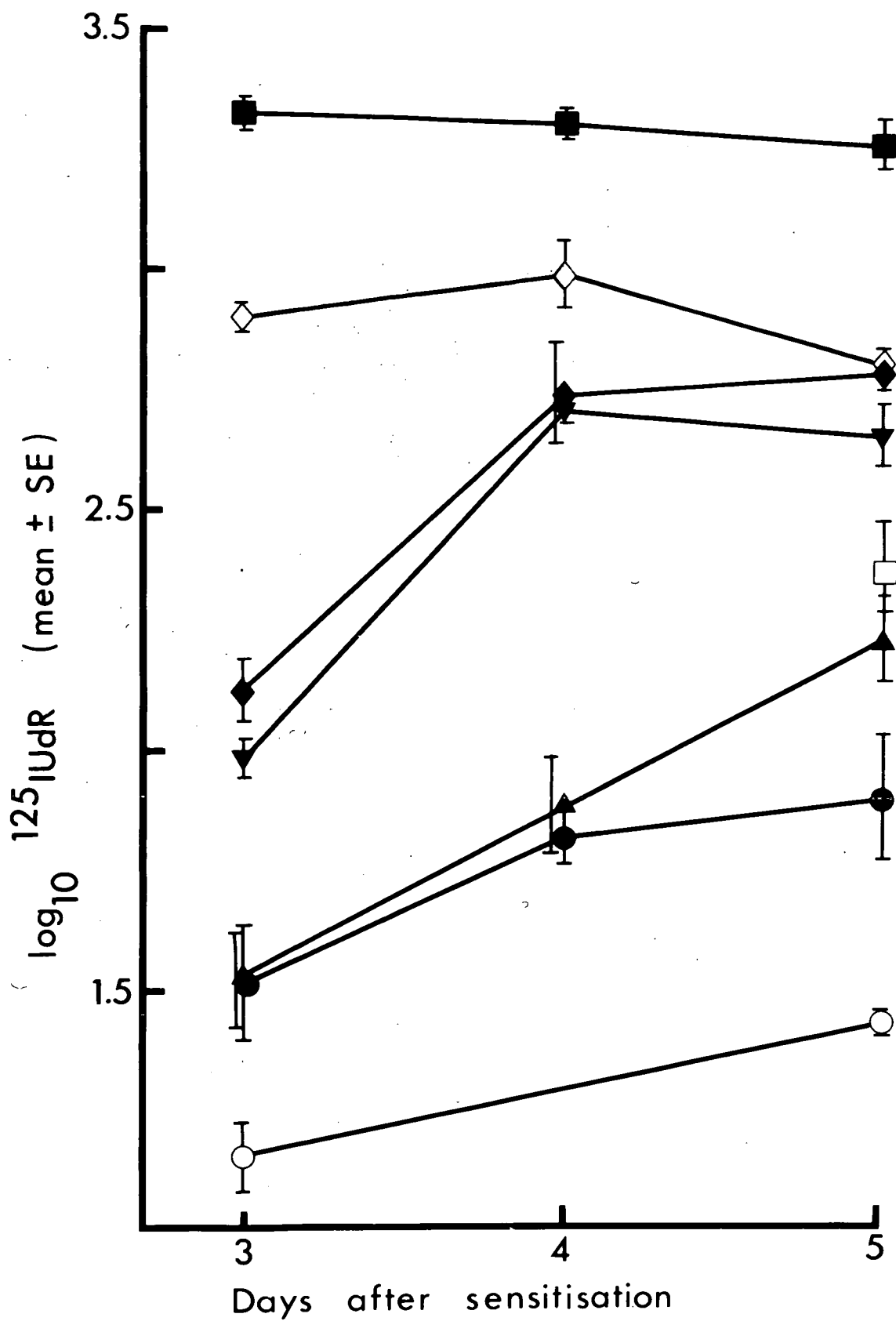


Fig. II.9

$^{125}$ IUdR incorporation by lymph nodes of irradiated recipients of  $10^6$  cortisone resistant thymocytes or  $10^6$  thymocytes, 3, 4 and 5 days after receiving oxazolone or alcohol.

The response in irradiated recipients of  $10^6$  cortisone resistant thymocytes and oxazolone  $\blacktriangle$  , of  $10^6$  cortisone resistant thymocytes and alcohol  $\triangle$  , of  $10^6$  thymocytes and oxazolone  $\blacklozenge$  , of  $10^6$  thymocytes and alcohol  $\lozenge$  , of saline and oxazolone  $\bullet$  , of saline and alcohol  $\circ$  ; in normal recipients of oxazolone  $\blacksquare$  .



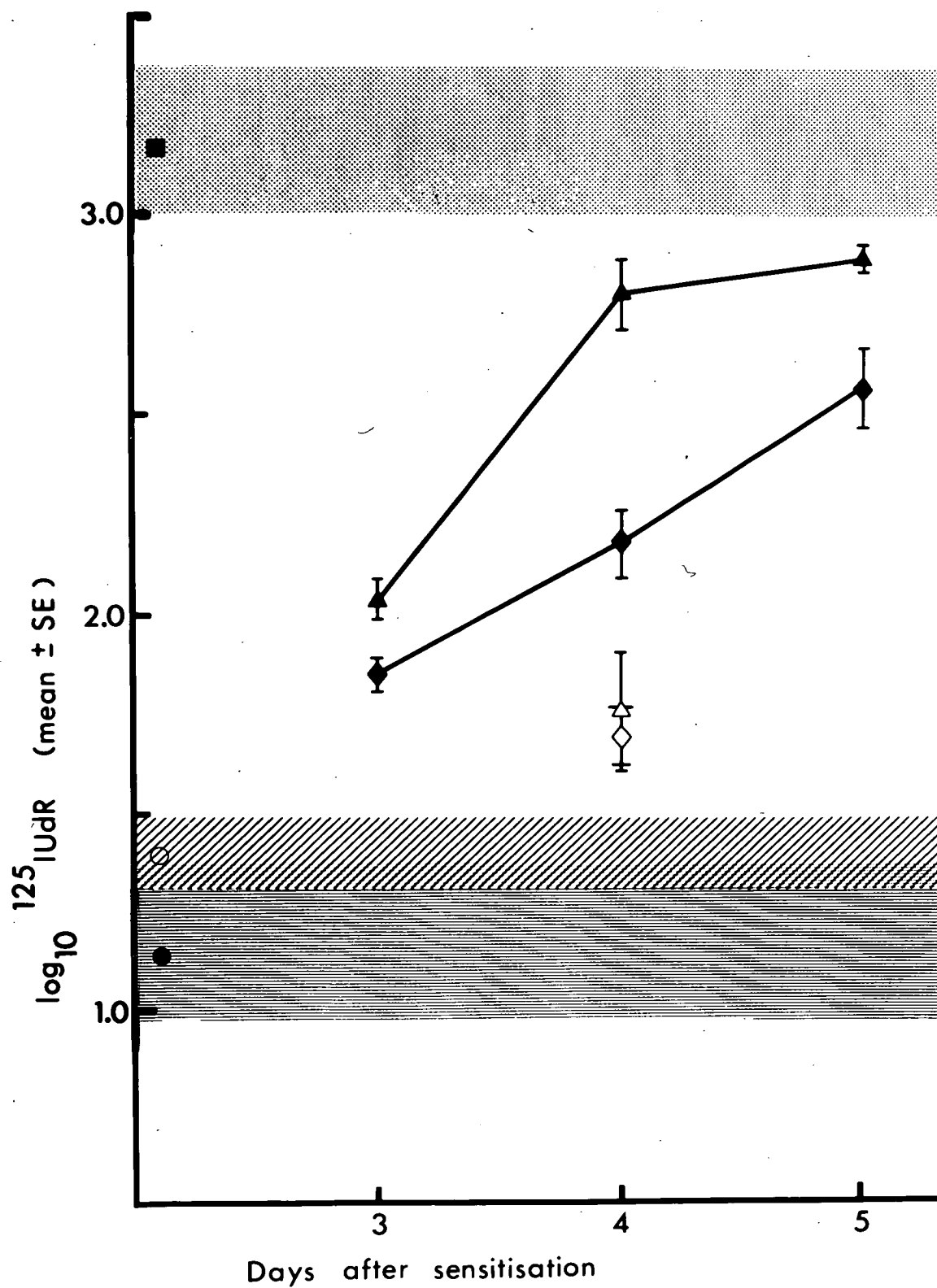


Fig. II.10

<sup>125</sup>IUdR incorporation by lymph nodes of irradiated recipients of different doses of lymph node cells, 4 days after receiving oxazolone or alcohol.

The response in irradiated recipients of lymph node cells and oxazolone ● , of lymph node cells and alcohol ○ , of saline and oxazolone ▲ , of saline and alcohol △ ; in normal recipients of oxazolone ■ or alcohol □ .

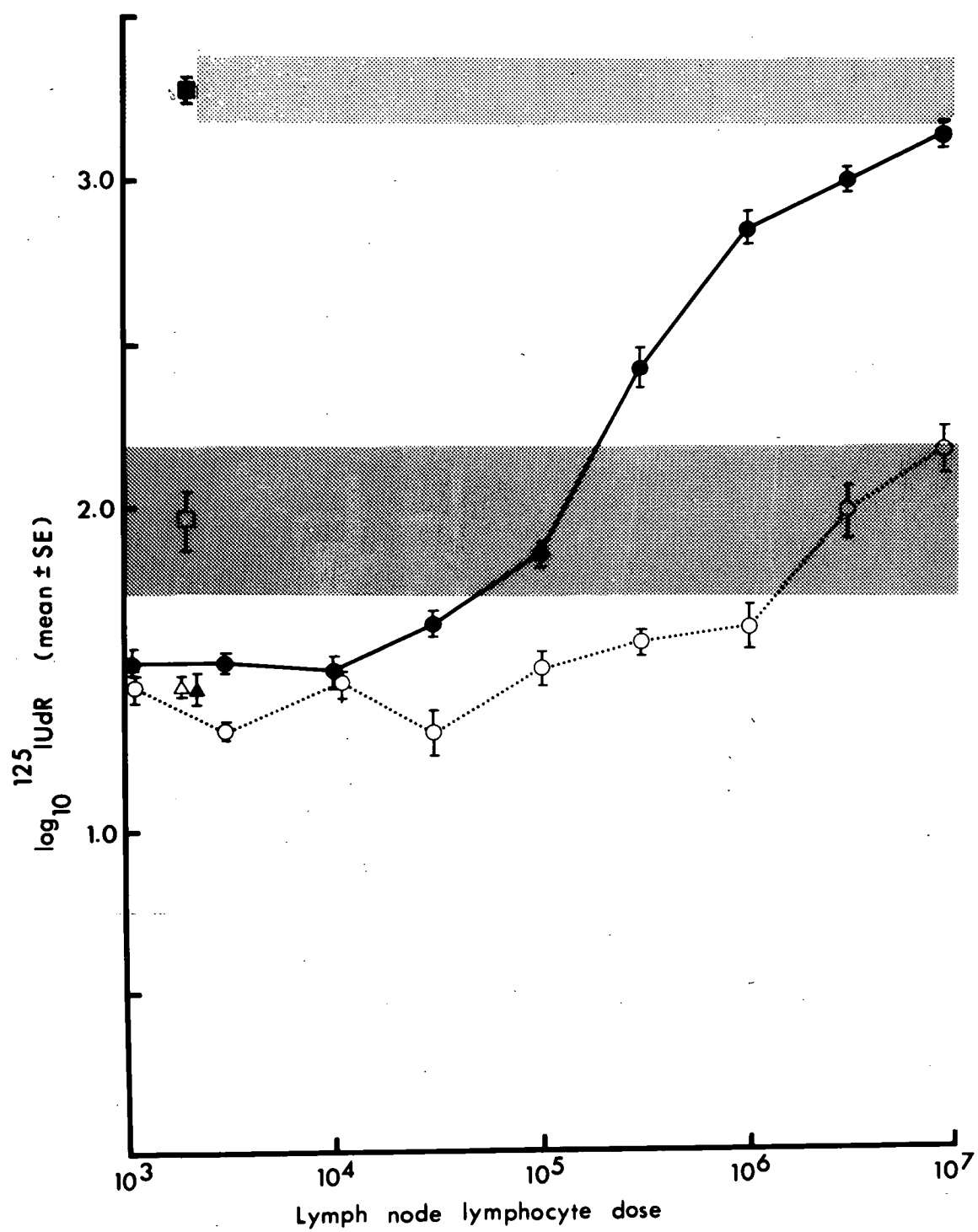


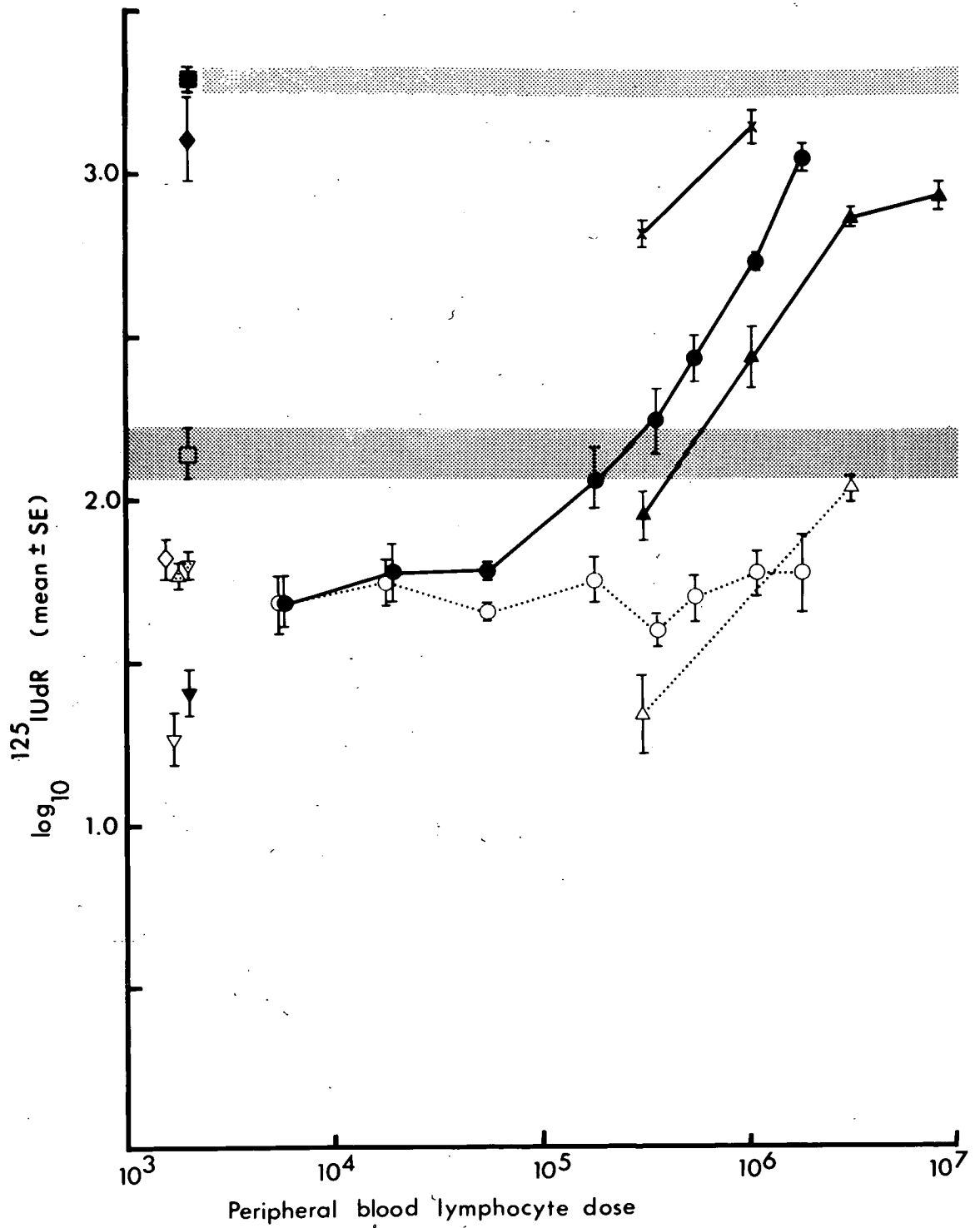
Fig. II.11

<sup>125</sup>IUdR incorporation by lymph nodes of irradiated recipients of different doses of peripheral blood lymphocytes, 4 and 5 days after receiving oxazolone or alcohol.

Exp.1: The response in irradiated recipients of peripheral blood lymphocytes (PBL) and oxazolone ● , of PBL and alcohol ○ , of saline and oxazolone △ , of saline and alcohol ▽ ; in normal recipients of oxazolone ■ or alcohol □ , 4 days after oxazolone.

Exp.2: The response in irradiated recipients of PBL and oxazolone ▲ , of PBL and alcohol △ , of saline and oxazolone ▼ , of saline and alcohol ▽ ; of normal recipients of oxazolone ◆ or alcohol ◇ 4 days after oxazolone.

The response in irradiated recipients of PBL and oxazolone 5 days after oxazolone X.



**Fig. II.12**

**<sup>125</sup>IUdR incorporation by lymph nodes of irradiated recipients of lymph node lymphocytes A, thymocytes B, cortisone resistant thymocytes C, lymph node lymphocytes and thymocytes A + B, lymph node lymphocytes and cortisone resistant thymocytes A + C in response to oxazolone (shaded) or alcohol (unshaded) 4 days after antigen administration.**

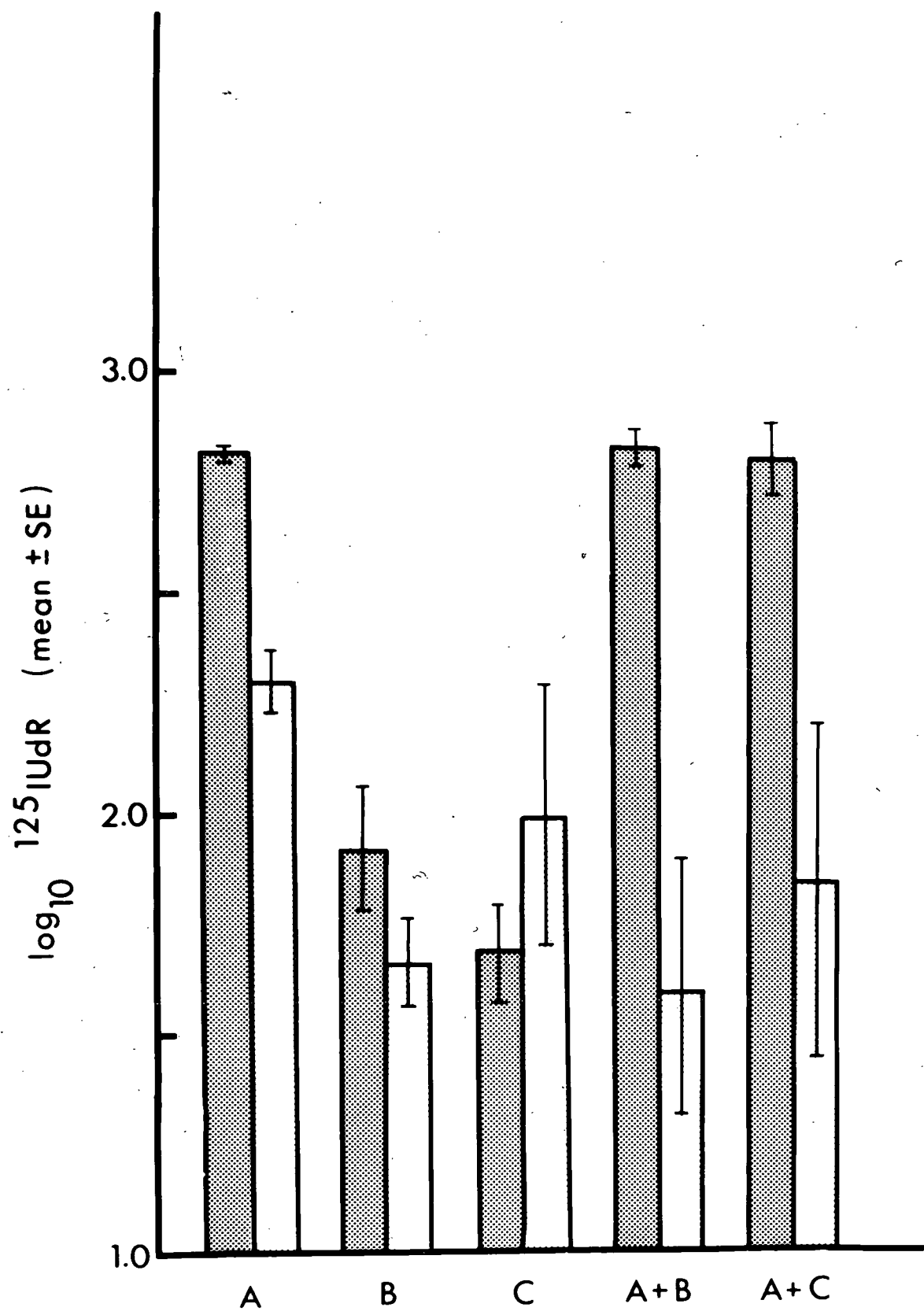


Fig. II.13

<sup>125</sup>IUdR incorporation by lymph nodes of irradiated recipients of anti-theta/<sup>treated</sup>immunised lymph node cells A, immunised lymph node cells treated with normal AKR serum B, anti-theta treated normal lymph node cells C, normal lymph node cells treated with normal AKR serum D, in response to oxazolone (shaded) or alcohol (unshaded), 4 days after antigen administration. The response of normal animals E, and irradiated recipients of saline F.



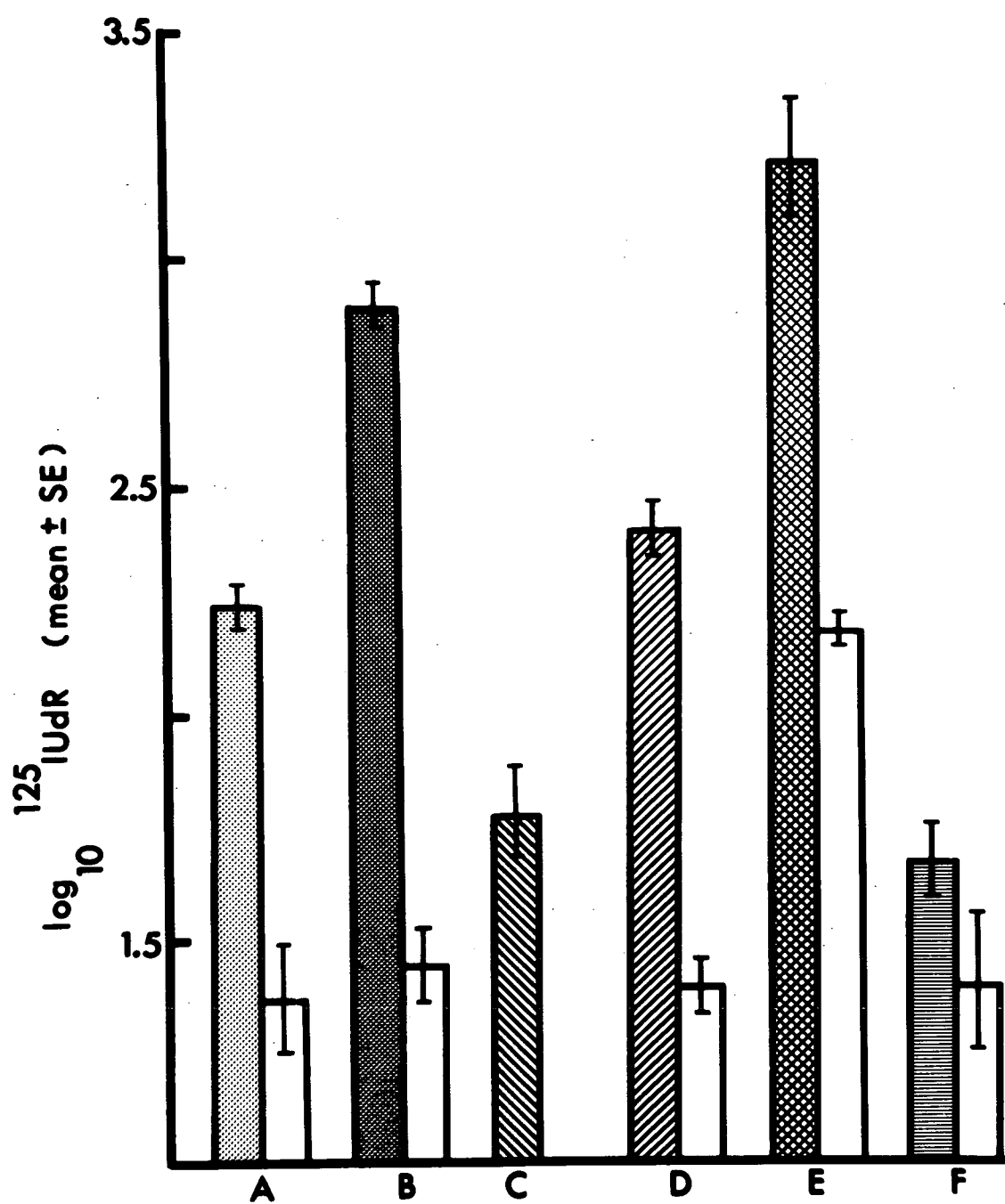


Fig. II.14

<sup>125</sup>IUdR incorporation by lymph nodes of irradiated recipients of different doses of thymocytes 3, 4 and 5 days after receiving 0.1mg of Ferritin or 0.05 mls of saline, injected s.c. into the right fore foot pad.

The response of irradiated recipients of  $10^6$  thymocytes  $\blacktriangle$  ,  $10^7$  thymocytes  $\blacklozenge$  ,  $3 \times 10^7$  thymocytes  $\bullet$  and  $10^8$  thymocytes  $\blacksquare$  to Ferritin. The response of irradiated recipients of  $10^6$  thymocytes  $\triangle$  and  $10^8$  thymocytes  $\square$  to saline.

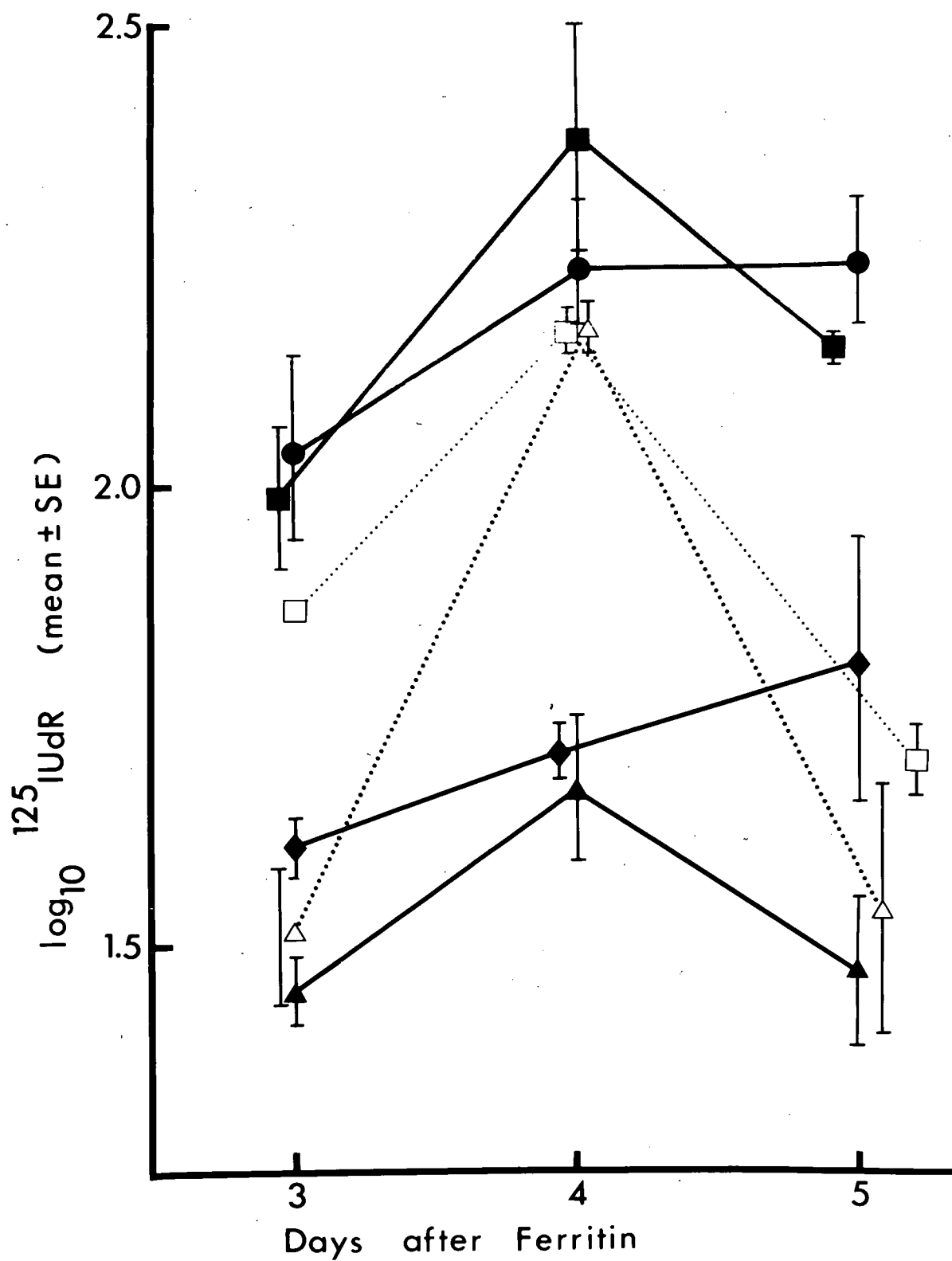


Fig. II.15

$^{125}$ IUdR incorporation by lymph nodes of irradiated recipients of different doses of thymocytes, 3, 4 and 5 days after receiving 2 $\mu$ gm of PVP or 0.05 mls of saline, injected s.c. into the right fore foot pad.

The response of irradiated recipients of  $3 \times 10^7$  thymocytes ● and  $10^8$  thymocytes ■ to PVP. The response of irradiated recipients of  $10^6$  thymocytes △ or  $10^8$  thymocytes □ to saline.

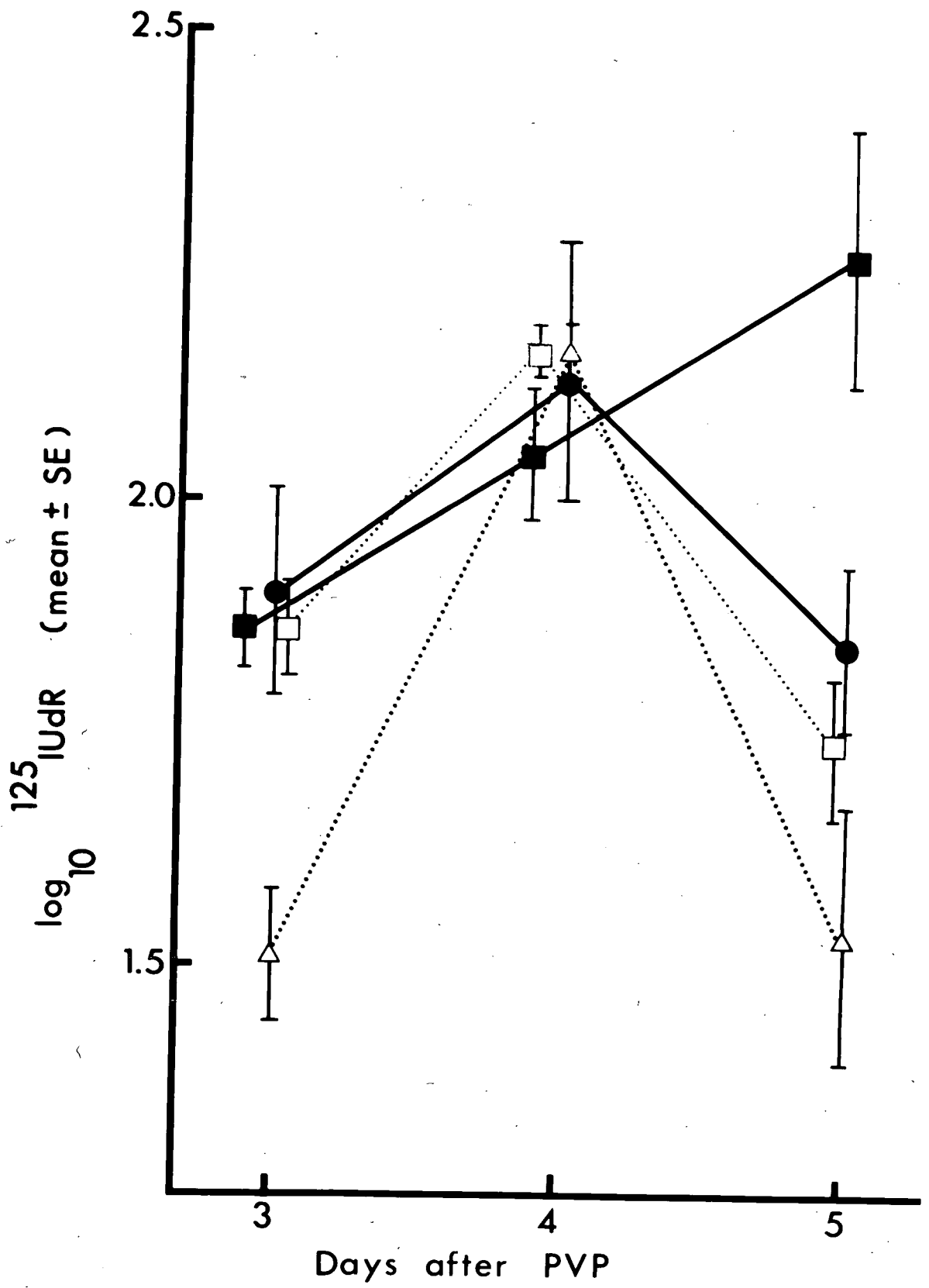


TABLE 11.1

Ratio of  $^{125}\text{I}$ UdR incorporated between the draining and contralateral lymph nodes in mice, 3 days after receiving different doses of oxazolone, which was painted on the left flank.

Oxazolone dose (mg)	Lymph nodes		
	Axillary and brachial	Inguinal	Popliteal
10	2.71	1.66	0.76
	5.24	0.75	0.14
	2.70	1.41	1.70
	2.47	0.66	0.80
	mean $\pm$ SD	3.28 $\pm$ 1.30	1.24 $\pm$ 0.49
1	12.05	0.82	0.83
	8.38	1.11	1.21
	10.32	1.57	0.86
		0.79	1.17
	mean $\pm$ SD	10.25 $\pm$ 1.84	1.07 $\pm$ 0.36
0.3	18.67	0.52	0.98
	8.74	1.28	2.72
	11.96	0.72	1.84
	22.38	0.88	1.50
	mean $\pm$ SD	15.44 $\pm$ 6.20	0.85 $\pm$ 0.32
0.1	14.89	00.69	3.60
	9.61	0.91	1.14
	9.11	0.93	1.77
	38.03	2.04	1.00
	mean $\pm$ SD	17.91 $\pm$ 13.66	1.15 $\pm$ 0.61

TABLE II.2

Ratio of  $^{125}$ IUdR incorporated between the draining and contralateral lymph nodes of mice, 3 days after receiving different doses of oxazolone, which was painted on the left hind foot pad.

Oxazolone dose (mg)	Lymph nodes		
	Axillary and brachial	Inguinal	Popliteal
10	0.98	2.20	3.15
	1.60	4.52	4.46
	1.23	2.63	4.98
		1.51	5.46
mean $\pm$ SD	1.27 $\pm$ 0.31	2.72 $\pm$ 1.29	4.52 $\pm$ 0.995
1	4.86	3.72	4.63
	1.43	10.09	1.97
	4.05	4.25	6.85
	1.82	10.17	5.52
mean $\pm$ SD	3.04 $\pm$ 1.67	7.06 $\pm$ 3.56	4.74 $\pm$ 2.06
0.3	1.13	12.23	1.41
	1.09	3.99	3.02
	0.99	2.87	2.56
	0.79	3.90	3.76
mean $\pm$ SD	1.01 $\pm$ 0.15	5.75 $\pm$ 4.35	2.69 $\pm$ 0.80
0.1	1.50	1.45	2.54
	1.30	4.57	22.33
	1.34	4.30	3.40
	1.93	9.92	1.09
mean $\pm$ SD	1.52 $\pm$ 0.29	5.06 $\pm$ 3.53	7.34 $\pm$ 10.04

TABLE II.3

<sup>125</sup>IUdR incorporation by lymph nodes of irradiated recipients of lymph node lymphocytes or peripheral blood lymphocytes from normal or adult-thymectomised mice, in response to oxazolone or alcohol, 3 and 4 days after antigen administration.

Exp.	Donor	Cell type, x 10 <sup>6</sup>	Oxazolone	Days after oxazolone. Mean <sup>125</sup> IUdR $\pm$ SE			
				Day 3	No. mice	Day 4	No. mice
I	ThX 7m	2.18, PBL	+	1.85 $\pm$ 0.04	5	2.39 $\pm$ 0.06	5
		1.0, LNC	+	2.09 $\pm$ 0.05	5	2.59 $\pm$ 0.16	5
	N 7m	2.72, PBL	+	2.34 $\pm$ 0.06	5	2.66 $\pm$ 0.08	5
		1.0, LNC	+	-	*	2.74 $\pm$ 0.104	3
	N 2.5m	2.22, PBL	+	2.47 $\pm$ 0.04	4	2.85 $\pm$ 0.05	5
		1.0, LNC	+	2.28 $\pm$ 0.05	4	2.91 $\pm$ 0.06	5
		2.22, PBL	-	1.88 $\pm$ 0.14	4	2.09 $\pm$ 0.15	5
		1.0, LNC	-	1.79 $\pm$ 0.06	5	2.10 $\pm$ 0.11	5
	ThX 9m	0.5, LNC	+	-	-	2.50 $\pm$ 0.038	6
		0.5, LNC	-	-	-	1.62 $\pm$ 0.09	6
II	N 9m	0.5, LNC	+	-	-	2.47 $\pm$ 0.03	6
		0.5, LNC	-	-	-	1.32 $\pm$ 0.14	6
	N 2.5m	0.5, LNC	+	-	-	2.33 $\pm$ 0.05	6
		0.5, LNC	-	-	-	1.78 $\pm$ 0.12	6
		No cells	+	-	-	1.84 $\pm$ 0.147	6
		No cells	-	-	-	1.50 $\pm$ 0.08	6

ThX = Thymectomised at four weeks of age

N = not thymectomised

7m = 7 months old

9m = 9 months old

2.5m = 2.5 months old

PBL = Peripheral Blood Lymphocyte

LNC = Lymph Node Cells



## DISCUSSION

1. Evaluation of the technique used to estimate cellular proliferation

Incorporation of  $^{125}\text{I}$ -labelled 5-Iodo-2'-Deoxyuridine ( $^{125}\text{I}$ -UdR) to measure DNA synthesis and hence provide an estimate of cell proliferation has been shown to be a sensitive and reproducible technique by various workers in this laboratory. The chemical and biological properties of  $^{125}\text{I}$ -UdR and the factors affecting its incorporation into DNA as a thymidine analogue were described by Prussoff (1963). It is incorporated into DNA and provides a stable label, that is, it is not removed from intact DNA in living cells (Commerford, 1965). The utilisation of  $^{125}\text{I}$ -UdR by various tissues of mice has been compared to the utilisation of tritiated thymidine (Fox & Prussoff, 1965; Feinendegen et al., 1966; Dethlefsen, 1970). Differences in the rate of incorporation, catabolism and reutilisation were detected between these two compounds. The main advantage of  $^{125}\text{I}$ -UdR is that reutilisation is reported to be negligible or non-existent (Hughes et al., 1964; Commerford, 1965; Feinendigen et al., 1966) or restricted to about 5% for the whole body (Hofer et al., 1969a; Hofer et al., 1969b; Dethlefsen, 1970). The other advantage, a practical one, is the relative ease of treating and counting tissues labelled with  $^{125}\text{I}$ -UdR. Due to the low energy of  $\beta$  particles, tissues which have been labelled with tritiated thymidine have to be homogenised or solubilised to disperse the cells, and mixed with scintillant before counting. Also, allowances have to be made for colour quenching which may occur when using blood or spleen.  $^{125}\text{I}$ -UdR

however is a gamma emitter and can be counted with no prior tissue preparation. The disadvantage of  $^{125}\text{I}$ -UdR is its potential toxicity (Jaffe & Prussoff, 1960; Dubbs & Kit, 1964; Morris & Cramer, 1966), the toxic effects can however be avoided by using  $^{125}\text{I}$ -UdR of high enough specific activity (Feinendegen et al., 1966; Fox & Lajtha, 1967). There is evidence that the iodine formed catabolically from  $^{125}\text{I}$ -UdR is probably not incorporated into proteins (Hughes et al., 1964; Fox & Prussoff, 1965). The technique which has been utilised in the present studies was first described by Hughes et al. (1964) who found that fluorodeoxyuridine (FUdR) by preventing the cellular synthesis of thymidine, greatly increased the incorporation of  $^{125}\text{I}$ -UdR.

$^{125}\text{I}$ -UdR has been used to estimate the amount of cellular loss from tumours (Dethlefsen, 1971), the proliferation of haemopoietic tissues (Bennet et al., 1968), the proliferation during a graft vs host response (Elkins, 1970; Gershon & Liebhaver, 1972; Gershon et al., 1972; Gershon et al., 1974), spleen and lymph node proliferation to bovine serum albumin and human serum albumin (Makela & Mitchison, 1965b), proliferation of spleen and lymph node cells in vivo to picryl chloride (Asherson & Barnes, 1973), proliferation of thymocytes to sheep erythrocytes (Gershon & Hencin, 1971; Kruger & Gershon, 1972), and to a variety of antigens (Kruger & Gershon, 1972). Pritchard and Micklem (1972) investigated the ability of athymic nu/nu mice to respond to oxazolone and compared the results obtained by measuring the response with  $^{125}\text{I}$ -UdR and measuring the change in weight between stimulated and unstimulated nodes. They found the method which used

$^{125}\text{I}$ -UdR produced results that were more consistent and probably more accurate than those obtained by weight measurements. When interpreting results that have involved the use of  $^{125}\text{I}$ -UdR it should be noted that not all DNA synthesis may be associated with cell division (Harris, 1973).

## 2. Parameters of the experimental system

Normal mice produce a high proliferative response to oxazolone as measured by the incorporation of  $^{125}\text{I}$ -UdR (Pritchard & Micklem, 1972). In order to investigate the ability of various types of lymphocytes to respond to oxazolone it was evident that a system that involved transfer of cell populations into irradiated recipients was necessary. The peak proliferation of T cells to oxazolone was shown to occur 3-5 days after the administration of the antigen (Davies et al., 1969b), thus in the present studies, responses were measured either on day 4, or on days 3, 4 and 5.

Experiments 1 and 2 were performed to discover the optimal dose of oxazolone to administer, the area of application and the timing of the irradiation relative to the timing of the administration of antigen. The results which are shown in Figs. II.1 and II.2 indicate that doses of oxazolone from 0.1 mg to 10 mg produce no difference in the amount of DNA synthesised during the response of the draining lymph nodes or spleen. Possibly all antigens have an optimal dose where increasing the dose no longer results in a concomitant increase in the response. Mikela and Mitchison (1965b) found this to be the case using bovine serum albumin and indeed discovered that high doses suppressed the response. Oxazolone appears to stimulate primarily those lymph nodes nearest the site of its application. An interesting feature of the

response is shown in Table II.1 where increasing the amount of oxazolone administered to the flank increased the DNA synthetic response in the distant nodes. Possible explanations for this phenomenon are that the distant nodes receive their stimulation either by excess oxazolone which has been transported internally or externally or by excess circulating sensitised lymphocytes. The question of central or peripheral sensitisation by skin grafts and contact sensitising agents remains unresolved. When oxazolone was painted on to the foot pad and subsequently covered, there was not such a pronounced effect of the increase in dose stimulating the distant nodes. This tends to imply that oxazolone was spread externally between the animals that were painted on the flank. The mice were not caged individually and contact between them would be probable. Experiments by Hall and Smith (1971) suggested that antigen from the site of contact sensitisation can be carried combined with soluble proteins in the afferent lymph, and pass through the draining lymph nodes, avoiding phagocytosis, into the efferent lymph and circulation. This might explain the effects of flank stimulation, but the same phenomenon did not occur when the foot pad was stimulated. Possibly the vascular or lymphatic connections between the inguinal and popliteal lymph nodes of the left and right sides are not direct enough to promote this effect.

The experiments in Chapter II required a system that would allow the maximum proliferative response, while at the same time minimising background proliferation. The results which were obtained by stimulating animals on the foot pad were variable, the ratios of stimulated to contralateral nodes were not as great as those which were produced by painting the thorax. It was therefore decided to routinely paint

the shaved area of the thorax and administer excess oxazolone, that is 5 mg.

Pilot experiments by A.C. Payne had indicated that administering oxazolone the day after irradiation and repopulation produced higher and more consistent results than performing all the procedures on one day. The results in Fig. 11.3 confirm this to be the case. It should be noted that there were no groups of mice which had been irradiated, repopulated and painted with alcohol and experience of this technique indicates that high responses can occasionally be due to non-specific proliferation. However, the fact that the same thymocyte suspension was used for all groups and the preliminary studies of A.C. Payne suggest that these results show a real advantage in delaying oxazolone administration for one day. Experiments have shown that lymphocytes, which have been injected intravenously become entangled in the fine capillary network of the lung during the first 2 hours, and possibly longer after their injection (Hall *et al.*, 1972). Oxazolone behaves similarly to many antigens (Ford, 1968; Zatz & Lance, 1971; Frost & Lance, 1974<sup>a</sup>) and causes circulating cells to become 'trapped' or retarded within the stimulated lymph node or spleen (as reported in Chapter IV) within 24 hours of antigen administration. It seems plausible that if for several hours cells are trapped in the lungs, the response in the lymph nodes may be delayed.

### 3. Kinetics of the T cell proliferative response to oxazolone

Experiments 3a, 3b, 3c and 3d were designed to investigate whether any factors regulate the magnitude of the proliferative response of T

cells to oxazolone and the nature of these factors.

If there were no control mechanisms which regulated the proliferative response of a lymphocyte population, increasing the number of potentially reactive cells would lead to a proportional increase in the response. T cells are a heterogeneous population with regard to maturity, membrane properties and possibly functions. In order to ascertain the possible role of different T cell populations in the proliferative response to oxazolone, the kinetics of various doses of lymphocytes from different anatomical compartments were investigated.

a) Thymocytes

The results which are shown in Figs. II.3-8 show that thymocytes, when injected into irradiated recipients, synthesise DNA in response to oxazolone. The irradiated recipients which had not been repopulated demonstrated that the DNA synthesis was due to injected thymocytes and the mice which had been given alcohol and thymocytes showed that the response was induced by oxazolone. The kinetics of the response are very similar to those given by thymocytes in response to histocompatibility antigens (Sprent & Miller, 1971; Gershon & Liebhaver, 1972) and sheep erythrocytes (Gershon & Hencin, 1971).

Within a certain range of doses, increasing the number of cells did not increase the magnitude of the proliferative response. A maximum response was reached with  $5 \times 10^7$  thymocytes (Fig. II.5 and II.8). This figure accords well with the results which were obtained from a similar experiment which investigated the proliferative response of CBA

thymocytes which had been injected into lethally irradiated (CBA x C57B1)  $F_1$  mice (Sprent & Miller, 1972a). The response was measured in the spleen by the uptake of tritiated thymidine. It was found that numbers of thymocytes between  $10^6$  -  $6 \times 10^7$  produced a linear increase in the response but it reached a plateau after  $6 \times 10^7$  cells.

It is conceivable that although doses of thymocytes above  $5 \times 10^7$  produce no greater response than lower doses on day 4, they may produce a greater response at a different time. Thus the response of  $3 \times 10^5$ ,  $3 \times 10^6$ ,  $10^7$  and  $7 \times 10^7$  thymocytes were measured at 3, 4 and 5 days after the application of oxazolone. The results (Fig. II.8) showed that:

- (i) the high dose of thymocytes produced their peak DNA synthetic response a day earlier than the lower doses.
- (ii) the DNA synthetic response of the high dose of thymocytes plateaued a day earlier than the lower doses, and possibly decreased thereafter.
- (iii) low doses of cells showed a lag phase before responding demonstrably.
- (iv) within a certain dose range, a twenty-fold increase in cell number did not produce any increase in proliferation.

The experimental system which was used by Gershon and Liebhaver (1972) to investigate the kinetics of the thymocyte response to histocompatibility antigens was similar to this system. They injected C3H thymocytes into lethally irradiated (C3H x DBA<sub>2</sub>)  $F_1$  recipients and measured the incorporation of  $^{125}$ I-UdR in the spleen and lymph nodes 1-5 days later.

The results showed features in common with those of the present studies. For example, they found that  $4.5 \times 10^7$  thymocytes produced their peak DNA synthetic response on day 3, and the response declined on day 5, in contrast to  $1.5 \times 10^7$  thymocytes which did not respond on day 3, but the amount of DNA synthesised increased between days 4 and 5. Gershon and Hencin (1971) found that increasing the thymocyte dose from  $5 \times 10^6$  to  $5 \times 10^7$  did not elevate the proliferative response to sheep erythrocytes.

The present studies, combined with the dose response studies described above (Gershon & Liebhaver, 1972; Gershon & Hencin, 1971) show that increasing the thymocyte dose by a multiple does not increase the amount of DNA which is synthesised by the cells in response to antigen by the same multiple. Moreover, within a certain dose range, increasing the number of thymocytes does not increase DNA synthesis at all. This observation implies the existence of a mechanism that regulates the magnitude of the proliferative response. Possible mechanisms are as follows:

- (i) Exhaustion of antigen; this is unlikely because more than enough antigen (5 mg) is present to produce the maximum response in normal animals.
- (ii) Exhaustion of some product necessary for DNA synthesis; this is unlikely in view of the fact that normal animals can support more DNA synthesis than that produced by the highest number of thymocytes, it may be possible however that irradiation depresses the concentration of nucleotides available.
- (iii) Negative feedback which inhibits T cells, produced by antibody or other B cell products; this may be a control mechanism which



operates in normal mice, but it seems unlikely it would operate in the irradiated thymocyte-repopulated animal. Nevertheless there could be radioresistant B cells that may exert an inhibitory effect.

- (iv) Migration of DNA synthesising cells from the lymph nodes; there is evidence that blast cells migrate from lymph nodes. For example Asherson et al (1973a) have suggested that blast cells migrate from lymph nodes 4 days after oxazolone, and showed that cells which have recently incorporated  $^{125}\text{I}$ -UdR migrated to areas of inflammation. Evidence that implies that thymocytes could be migrating in the present experiments stems from the findings of Sprent & Miller (1972a) that blast cells appear in the thoracic duct lymph of mice which had been given allogeneic thymus cells 4-5 days previously. It is thus possible that blast cells, which were produced as a result of thymocytes responding to oxazolone may be leaving the draining node 4 and 5 days after the application of antigen. However, on the assumption that the same proportion of blast cells leave the nodes from animals which were injected with low numbers of thymocytes as leave the nodes of animals containing relatively high numbers of thymocytes, this does not readily explain why the response plateaus, a phenomenon only observed with high doses of cells. To consider cell migration a plausible explanation, it is necessary to assume or postulate that migration can occur only after a certain number of cells have proliferated or have been produced.

- (v) Thymocytes are a heterogeneous population of cells, and it has been suggested that they may contain a population with suppressor activities (Chiller et al., 1971; Elliot, 1973). The kinetics of the thymocyte proliferative response to histocompatibility antigens was explained by the suppressor activities of certain populations (Gershon & Liebhaver, 1972).

b) Mature T cells

The majority of thymocytes are immature (Blomgren & Andersson, 1969) and the possibility that a suppressive effect is mediated by immature cells that normally perhaps never leave the thymus was investigated. Thus the dose response kinetics of cortisone resistant thymocytes, lymph node lymphocytes and peripheral blood lymphocytes were investigated.

The responses of these cell suspensions were measured only in the lymph nodes. The results in Figs. II.6 and II.7 indicate that very little proliferation occurs in the spleen, in a transfer situation. It is conceivable that the splenic response is delayed due to the accumulation and temporary trapping of injected cells within the stimulated lymph nodes. Asherson and Barnes (1973) found that the spleen in normal mice produced three peaks of DNA synthesis in response to picryl chloride, on days 2, 5 and 8. They suggested the day 5 result was due to cells which had migrated from the lymph nodes. It may therefore be possible that if irradiated recipients could be maintained long enough, a response would be detected in the spleen.

### 1) Cortisone resistant thymocytes

The DNA synthetic response of lymph nodes from mice which had been irradiated and repopulated with  $10^6$  thymocytes or cortisone resistant thymocytes was compared, (Fig. II.9) 3, 4 and 5 days after the application of oxazolone. It can be seen that the kinetics and magnitude of DNA synthesis produced by  $10^6$  cortisone resistant thymocytes is similar to that produced by  $10^7$  thymocytes (Fig. II.3). These results suggest that cortisone resistant thymocytes are, cell for cell, about ten times as active as normal thymocytes in responding to oxazolone.

It is to be expected that cortisone resistant thymocytes represent a large proportion of the thymocytes which proliferate in response to oxazolone. They account for a large proportion of immunocompetent cells in the thymus (reviewed by Claman, 1972). Cortisone resistant thymocytes were found to be ten times as effective as thymocytes in producing splenomegally (Blomgren & Anderson, 1969) and thirty times as effective as thymocytes in the in vitro response to PHA and allogeneic cells (Blomgren & Svedmyr, 1971). The kinetics of the response to oxazolone, presented in these studies, is similar to those found by Blomgren and Svedmyr (1971). It is interesting that the amount of  $^{125}\text{I}$ -UdR which was incorporated by mice which had received  $10^6$  cortisone resistant thymocytes, and were giving a 5 day response to oxazolone ( $2.6 \pm 0.09$ ) was similar to the amount incorporated by mice which had received  $10^7$  thymocytes ( $2.75 \pm 0.1$ ). These figures represent approximately the peak performance achieved by thymocytes in these series of experiments, that is when  $7 \times 10^7$  thymocytes produced a

$^{125}$ I-UdR uptake of  $2.98 \pm 0.08$ . Thus it appears that the proliferative response fails to increase, or declines when a certain amount of proliferation has occurred. Sprent and Miller (1972a) found the proliferative response of thymocytes and cortisone resistant thymocytes in allogeneic recipients levelled off after similar amounts of  $^3$ H-thymidine had been incorporated.

#### 11) Lymph node and peripheral blood cells

The response of various numbers of lymph node and peripheral blood lymphocytes to oxazolone were examined to ascertain whether these cell types also produce a plateau of responsiveness at high doses.

The response of mice which had received lymph node cells (Fig. II.10) increased in an injected dose range of  $3 \times 10^4 - 10^6$  cells, but thereafter the response levelled off. Peripheral blood lymphocytes showed (Fig. II.11) a dose dependent increase between  $5 \times 10^4$  and  $3 \times 10^6$  cells, but the response which was produced by  $10^7$  cells was no greater than to  $3 \times 10^6$  cells. Thus a prima facie interpretation of these observations is that there is a levelling off of the response at high doses. However a factor which limits the interpretation of these results is that the DNA synthetic response was only measured on day 4. As can be seen from Fig. II.11, where the response of animals which had been injected with  $3 \times 10^5$  and  $10^6$  cells was measured on days 4 and 5, there was an increase in the amount of DNA syntheses between days 4 and 5.

A possible reference point with which to compare these results, is the amount of  $^{125}$ I-UdR which was incorporated by normal unirradiated mice

4 days after oxazolone. The peak response of the mice which had received the largest numbers of thymocytes was significantly less than normal. Animals which received cortisone resistant thymocytes and peripheral blood cells did however attain the normal level. Previous experiments by N. Anderson and H.S. Micklem (pers. comm.) which utilised lymph node lymphocytes in dose response experiments, indicated that animals which have been repopulated with these cells may produce a proliferative response to oxazolone as great as the normal response. The response of lymph node cells may however include proliferating B cells. Davies et al. (1969b) utilised thymectomised mice which had been injected with bone-marrow cells and  $3 \times 10^7$  thymocytes. Fifty days later they were painted with oxazolone. It was found that on day 4 of the response, over 75% of dividing cells in the draining nodes were of thymic origin.

It would be difficult to conclude on the basis of the present studies that there is any real difference in the kinetics of the proliferative responses of lymph node cells, peripheral blood lymphocytes, cortisone resistant thymocytes or thymocytes. These results do not provide evidence for the existence of qualitatively different T cell populations that either suppress or enhance other T cells in their proliferative response to oxazolone. A possible and perhaps simple interpretation of these results would be that:

- (i) the amount of  $^{125}\text{I}$ -UdR which is incorporated reflects the number of responsive cells which are present.
- (ii) a mechanism operates which prevents further synthesis of DNA when a certain number of cells have divided.

The drawback of these experiments is that responses were measured on only one or three days. Exploring the system over a longer time period may well reveal patterns of responsiveness that distinguish these cell populations qualitatively as well as quantitatively. For example, Sprent and Miller (1972a) investigated the pattern of the proliferative response of thymocytes, cortisone resistant thymocytes and thoracic duct cells in the spleens of irradiated allogeneic recipients over 10 days and concluded that the cell suspensions contained qualitatively different cell populations.

A possible interpretation of the difference between the profile of the thymocyte response to oxazolone and that of the other cell suspensions may be simply that 95% of a thymocyte cell suspension are immature, non-responsive cells. There is evidence however to suggest that the thymus has suppressive effects in some circumstances (as described in Chapter II) and the possibility that some of the 'non-responsive' cells may be suppressing the response should be considered for two reasons:

- (i) The peak response of the highest number of thymocytes is well below the level of proliferation which was achieved by unirradiated mice. This is not true of cortisone resistant thymocytes or peripheral blood cells.
- (ii) The plateau part of the thymocyte response which is shown in Fig. II.5 is a more convincing plateau than those found using other cell types.

The results from Experiments 3a, 3b, 3c and 3d suggest the existence of a homeostatic mechanism which controls the amount of cell division which occurs in response to oxazolone. What bearing do these results have on the response in normal mice and its regulation? They show the peak DNA synthetic response of lymphocytes in the transfer situation to occur 4 days after oxazolone, that is one day later than the peak response in normal mice (Pritchard & Micklem, 1972). This may be due to the time taken for injected cells to localise. The normal mouse has been found to show intense paracortical hyperplasia one day after oxazolone, this reaches a maximum on days 3 and 4, and thereafter declines (Oort & Turk, 1965; Parrott & de Sousa, 1966). The early phase of proliferation is mainly T cells. The B cells produce their maximum proliferation on days 5 and 6 (Davies et al., 1969b). Thus T cells proliferate to oxazolone in irradiated and normal mice with maximum activity on days 3 and 4, and thereafter activity declines. This pattern of T cell proliferation may be characteristic for contact sensitisation and allograft responses. For example, animals which were painted with picryl chloride produced a maximum DNA synthetic response in their draining lymph nodes on day 3, and the response declines on day 5, as measured in vivo (Asherson & Barnes, 1973) and in vitro (Barnes, 1973). Thymocytes also showed a similar pattern of mitosis when undergoing a graft vs host response (Gershon & Liebhaver, 1972; Sprent & Miller, 1972c). Whether this pattern of mitotic activity is characteristic of all T cells and induced by all T cell antigens is unknown. In mice which had been neonatally thymectomised, irradiated, bone-marrow reconstituted and given a thymus graft, lymph node cells of thymic origin were shown to proliferate to

a variety of antigens (Carter et al., 1969). The response to oxazolone showed kinetics similar to the present findings. The response to sheep erythrocytes peaked sharply on day 3 and fell between days 3 and 4. The response to bovine gamma globulin peaked on day 4 and fell on day 5. The response to bovine serum albumin rose gradually between days 3 to 6, and the response was not followed thereafter. It has been suggested (Gershon & Liebhaver, 1972; Gershon, 1974) that the kinetics of the T cell response to histocompatibility antigens in irradiated recipients is produced by interactions between T cell populations of a suppressive nature, and that moreover, interactions of this type operate in the normal animals and serve to regulate the response.

4. Experiment to investigate the existence of suppressive or potentiating interactions between T cells, affecting the response to oxazolone

There is evidence that in certain circumstances thymocytes, cortisone resistant thymocytes and thymocytes from immunised recipients ('educated thymocytes') can either potentiate or depress the proliferation of other T cells. Thus Cantor (1972b) found that  $5 \times 10^5$  lymph node cells and  $5 \times 10^6$  thymus cells gave a graft vs host response greater than could be accounted for by the sum of the responses measured by splenomegally. On the other hand, Gershon et al. (1972) showed that  $1 \times 10^7$  T cells from mice which had been immunised to sheep erythrocytes, when mixed with  $10^7$  thymocytes and transferred into an irradiated recipient, depressed the response to sheep red cells to a level below that attained by educated T cells independently.



If synergistic and suppressive response between T cell populations could be detected in the above systems, they might be detected in the system used in the present studies. Experiment 4, Fig. II.12, shows the effect on the proliferative response to oxazolone of mixing  $5 \times 10^5$  lymph node cells with thymocytes or with cortisone resistant thymocytes. The results did not show any sign of synergistic or suppressive effects resulting from mixing cell suspensions. They are however difficult to interpret for two reasons:

- (i) mice which had been injected with cortisone resistant thymocytes or thymocytes gave no appreciable response, despite the results from earlier experiments (Fig. 4 and Fig. 9) where the same doses produced significant responses;
- (ii) mice which had been injected with mixed cell suspensions, and painted with alcohol, showed lower background proliferation than recipients of lymph node cells which had been painted with alcohol.

Thus although there was no absolute difference in the amount of  $^{125}\text{I-UdR}$  incorporated by recipients of lymph node cells and recipients of the cell mixtures, comparison with the groups of animals that had received alcohol might suggest that the recipients of cell mixtures produced a greater specific response to oxazolone than could be accounted for by the sum of the responses.

This type of experiment should be repeated several times with different cell combinations and the effect of cell number should be properly controlled, in order to attain an acceptable result that conclusively shows suppression or synergy.

### 5. Factor of immunisation of oxazolone

Antigens differ in their ability to stimulate T cells to divide, thus oxazolone (Carter et al., 1969) and histocompatibility antigens appear to stimulate large numbers of T cells. In contrast there is little T cell proliferative activity which is induced by salmonella H-antigens (Carter et al., 1969; Davies et al., 1970), pneumococcal polysaccharide III (Davies et al., 1970; Kruger & Gershon, 1971) or polyvinylpyrrolidone (Kruger & Gershon, 1972). It could be postulated that the ability to stimulate large numbers of T cells into mitosis is in some way correlated with the ability to produce delayed hypersensitivity and contact sensitisation. It seems plausible therefore that the T cell proliferative response to oxazolone may have some features in common with the proliferative response to histocompatibility antigens. This hypothesis was explored in experiment 5, which was designed to investigate whether lymphocytes from a mouse which had been previously sensitised to oxazolone would produce a heightened proliferative response to that antigen.

Immunisation against strong transplantation antigens fails to elicit a heightened secondary response measured by graft vs host activity, in contrast to the memory response which is produced by immunising across weak histocompatibility differences (Simonsen, 1962; Ford & Simonsen, 1971). This could either be because the proportion of cells reactive to a strong transplantation antigen is so high in the unimmunised animal that the maximum possible increase would only slightly elevate the graft vs host response (Simonsen, 1962) or (a hypothesis postulated by Ford & Gowans, 1967, and supported by the experiments

of Rolstad and Ford, 1974) because proliferation of lymphocytes to strong transplantation antigens is directed more towards producing cytotoxic effector cells rather than cells able to give a secondary proliferative response. Some of the products of T cell proliferation to oxazolone are cytotoxic in vivo (Asherson et al., 1973a). In experiment 5, lymphocytes were obtained from the lymph nodes of mice 14 days after they had been sensitised to oxazolone. Day 14 was chosen to ensure that few blast cells would be present and by day 14 the products of proliferation appear to be well disseminated throughout the body (Asherson & Zembala, 1973). The batch of anti-theta serum used was not very efficient in killing only 28% of the lymph node cells, it did however kill 66% of cells from immunised mice; possibly this is because primed cells have more theta antigen on their surface, or because they are more susceptible to lysis for some other reason. Kerbal (1974) found that blast cells removed from animals 4 days after oxazolone treatment were more susceptible to anti-theta than normal cells. Despite the apparent inefficiency of the anti-theta serum, it depressed considerably the ability of normal lymph node cells to transfer responsiveness to oxazolone. Mice which had received cells from immunised donors incorporated significantly higher amounts of  $^{125}\text{I}$ -UdR in their draining lymph nodes than did recipients of normal lymph node cells. It is reassuring that the recipients of normal lymph node cells incorporated as much  $^{125}\text{I}$ -UdR as would be predicted from the lymph node dose response curve shown in Fig. II.10. Thus there was a real specific elevation of the magnitude of proliferation produced by primed cells. The Factor of Immunisation for transplantation antigens has been calculated by constructing dose response curves of

normal and immunised cell populations, and comparing the number of normal cells to immunised cells which are necessary to produce a certain level of response (Ford & Simonsen, 1971). Unfortunately, this experiment was not done in the present study, but using the lymph node dose response curve of Fig. II.10, it is possible to estimate approximately that the amount of isotope which was incorporated by  $3 \times 10^5$  cells from immunised donors ( $2.9 \pm 0.05$ ) would require more than  $10^6$  normal lymph node cells, to achieve the same level of incorporation. It may be possible to increase the Factor of Immunisation for oxazolone by experimenting with different immunisation schedules. Anti-theta serum depressed the ability of primed cells to transfer the proliferative response, but to a level considerably above the amount of  $^{125}\text{I-UdR}$  which was incorporated by recipients of anti-theta treated normal lymph node cells. The spurious quality of the anti-theta serum precludes any definite statement, but it seems possible that B cells formed part of the immune population, and proliferated in response to oxazolone.

6. Effect of adult thymectomy on the ability of lymphocytes to transfer the proliferative response to oxazolone

Extirpation of the thymus in early life results in severe impairment of cell-mediated immunity (Miller, 1961; Arnason et al., 1962) and some humoral antibody responses (Miller & Osoba, 1967). It results in severe depletion of the number of lymphocytes found in the thoracic duct and blood (Miller & Mitchell, 1967; Agnew, 1967) and in the T dependent areas of lymph nodes and spleen (Parrott et al., 1966). Adult thymectomy however does not produce visible immunodeficiencies until at least six months after thymectomy. For example, mice that

had been thymectomised when they were 4-8 weeks old, showed a depressed ability to mediate graft vs host responses some seven months later (Taylor, 1965; Miller, 1965) and their ability to produce haemolysin to sheep erythrocytes was not severely depleted until 11 months after thymectomy (Metcalf, 1965). Adult thymectomy impairs recovery from procedures which in normal mice cause mild and reversible damage to the immune system such as a single dose of 35OR (Miller, 1962) or administration of a single dose of Antilymphocytic sera (Jeejeebhoy, 1965). It would be expected to affect the lymphocyte population after 6 months in the following ways:

- (i) The short-lived T cell population would be depleted. This may be apparent after 1-2 weeks (Cantor, 1972c).
- (ii) The pool of long-lived, recirculating lymphocytes may be depleted. As the long-lived cells outlive their life-span, or become depleted due to excessive use or possibly damage, they will not be replaced by the thymus.

It would therefore be expected to find some depression in the ability of lymphocytes from thymectomised mice to transfer a day 4 proliferative response to oxazolone.

The first experiment showed that there were fewer lymphocytes in the blood of 7 month old thymectomised mice ( $5.45 \times 10^6$  per ml) than in the blood of 7 month old normal mice ( $6.8 \times 10^6$  per ml). It is however possible that the latter figure is abnormally high as young mice gave a blood count of  $5.55 \times 10^6$  per ml. The response of mice which had been reconstituted with peripheral blood cells from thymectomised donors was delayed rather than completely suppressed.

Thus 3 days after oxazolone they gave no significant response, but at day 4, the amount of DNA synthesised in recipients of oxazolone was significantly greater than the mice which had received alcohol. The delaying effect was not due to age, because on day 3, the lymphocytes from the thymectomised mice were, cell number for cell number, less responsive than the lymphocytes from 7 month old mice. 4 days after oxazolone there was no appreciable difference in the ability of peripheral blood cells from thymectomised or normal mice to transfer the response. The dose response experiments in these studies (experiments II.3) showed that low doses of cells are characterised by a lag phase before responding. These results do therefore suggest that the blood of adult thymectomised mice contains fewer cells able to proliferate in response to oxazolone than does the blood of normal mice of the same age. Miller et al. (1963) found that the ratio of small cells/large cells in the blood, dropped from 5.6 in normal mice to 2.2 in mice which had been adult thymectomised, suggesting a lack of the immunocompetent small lymphocyte in the thymectomised animal. It should be noted that these results may be distorted due to the fact that 1/8 of the blood which was obtained from thymectomised donors was contributed by a donor with one thymus lobe.

Lymph node lymphocytes from thymectomised and 7 month old donors transferred the proliferative response to oxazolone as well as lymphocytes from young mice. Possibly blood is depleted of immunocompetent cells sooner and to a greater extent than are the lymph nodes. Adult thymectomy has been shown to produce depletion of lymphocytes in the T dependent areas of lymph nodes and spleen.

(Miller et al., 1963; Metcalf, 1960; Miller, 1965). Presumably the lymph nodes of thymectomised mice contain a higher proportion of B cells than do normal lymph nodes, and as B cells probably proliferate in response to oxazolone on day 4 (Davies et al., 1969b), the present experimental system can probably not detect the T cell deficiency. It is also possible that, because experiments using adult thymectomised mice tend to be variable (Miller, 1965), if more donors had been used in the present studies a T cell deficiency in the lymph nodes might have been detected.

The second experiment utilised mice which had been left 8 months after thymectomy. The absence of any difference in the ability of lymph node cells from thymectomised and normal donors to transfer the response to oxazolone accords with the results from the first experiment. Other workers in this laboratory have investigated the ability of old and thymectomised mice to transfer responses to oxazolone into irradiated recipients (N. Anderson & H.S. Micklem, pers. comm.). They found that  $10^6$  lymph node cells, spleen cells and peripheral blood cells from 29 month old mice were significantly depressed in their ability to transfer responses to oxazolone compared to 3 month old mice (P 0.001; 0.02; 0.001; respectively). In another experiment however, they failed to detect any difference in the responsiveness of lymph node cells, spleen cells and peripheral blood cells from normal 30 month old mice and 26 month old mice which had been adult thymectomised at one month of age.

These results, considered with the present studies, suggest that either:

- (i) the ability of lymph node cells to respond to oxazolone is not diminished by adult thymectomy, or
- (ii) the ability of lymph node cells to respond to oxazolone is diminished by thymectomy, but the effect is obscured by ageing.

The first hypothesis is possible if sufficient B cells proliferate to oxazolone on day 4 to obscure the T cell deficiency. The second suggestion implies that any depression in the response to oxazolone which is caused by thymectomy is not apparent until at least 8 months after thymectomy.

To conclude, these results suggest:

- (i) There is a population of lymphocytes in the blood that is responsive to oxazolone, and that is depleted within 6 months by adult thymectomy.
- (ii) There is a population of lymphocytes situated within the lymph nodes, that are responsive to oxazolone and that are not depleted within 8 months by adult thymectomy. They could be long-lived T cells or B cells.

#### 7. The proliferative response of thymocytes to ferritin and PVP

This experiment (Figs. II.14 and II.15) was designed to investigate the following:

- (i) The present studies (II.3) have shown that the proliferative response to oxazolone fails to increase in magnitude after about  $10^8$  thymocytes and  $10^6$  lymph node cells have responded.



Does this phenomenon occur using antigens other than oxazolone?

- (ii) If a decline in the amount of proliferation does occur, does it happen when a certain amount of DNA has been synthesised, or when a certain number of thymocytes are present?
- (iii) Polyvinylpyrrolidone (PVP) has been reported to be a T independent antigen (Fahey et al., 1965). Kruger and Gershon (1972) investigated whether  $5 \times 10^7$  thymocytes could transfer the proliferative response to PVP into irradiated recipients, the response was measured in the spleen. The experiment was repeated using  $5 \times 10^7$  and  $10^8$  thymocytes and measuring the response in the lymph nodes.

In order to investigate points (i) and (ii) it was decided to use an antigen which produces moderate stimulation of T cells. From the results of Kruger and Gershon (1972), ferritin produced approximately as much stimulation of T cells as did sheep erythrocytes. It is difficult to determine how much of the DNA which was synthesised by recipients of thymocytes and ferritin (Fig. II.14) was a specific response to ferritin, because the amount of DNA synthesised in recipients of thymocytes in response to saline is so high. Mice which had been repopulated with  $10^8$  thymocytes did not produce a greater response than those which had received  $5 \times 10^7$  thymocytes; the greatest incorporation of  $^{125}\text{I}$ -UdR ( $2.36 \pm 0.1$ ) was considerably lower than the maximum that was incorporated by thymocytes that were responding to oxazolone (Fig. II.8,  $2.98 \pm 0.07$ ). The latter observation could form the basis of a proposition that a 'switch-off mechanism' does occur during the response to ferritin and it occurs after considerably less DNA has been

synthesised, than in the oxazolone response. This would imply that the mechanism which controls the amount of cell division which occurs during a response is mediated not by effectors which respond to some critical level of proliferation independent of the antigen, but by effectors which are antigen dependent. However, because of the high responses of mice which received saline and no antigen, it is pertinent to question whether there is any response to ferritin in Fig. II.14 0.1 mg of ferritin was injected without adjuvant, as described by Kruger and Gershon (1972). They did apparently have some difficulty in dissolving it, thus possibly the ferritin was not adequately dissolved in the present experiment.

Kruger and Gershon (1972) reported that irradiated mice which received  $5 \times 10^7$  thymocytes and PVP, failed to incorporate  $^{125}\text{I}$ -UdR in response to the antigen. The results in Fig. II.15 show that irradiated recipients of  $10^8$  thymocytes and PVP incorporated significantly more  $^{125}\text{I}$ -UdR than did irradiated recipients of thymocytes and saline. The response was measured 5 days after the antigen and the standard errors were large. These results may suggest that there is no such phenomenon as a complete inability to stimulate T cells. They may however be due to experimental error, or even the presence of some B cells in the thymocyte population.

To obtain a meaningful picture of the kinetics of T cell proliferation and its antigen dependence, experiments of this type could be performed using a variety of antigens. It is obviously important to measure the response over several days and include non-stimulated control groups of animals.

### CHAPTER III

#### THE ANTIBODY RESPONSE TO OXAZOLONE

##### INTRODUCTION

It is established that mice respond to transplantation antigens by the production of at least two types of effector cells, cells that produce and release antibody and cells which display a cytotoxic activity against target cells carrying the sensitising antigen (Brunner & Cerottini, 1971). Antibodies have been detected in the sera of mice possessing skin allografts (Amos et al., 1954; Boraker & Hildemann, 1965; Rolstad et al., 1974) and they have also been detected by the presence of plaque forming cells (Hildemann & Pinkerton, 1966; Hildemann, 1967; Cerottini et al., 1971) and rosette forming cells (Micklen et al., 1970). Despite the suggestive correspondence between the time course appearance of plaque forming cells and the time of acute graft rejection (Hildemann, 1967) there is no direct evidence that allo-antibodies play a role in initiating graft rejection. Thus the passive transfer of the ability to reject skin allografts is achieved by lymphoid tissue and immunised sera are ineffective (Medawar, 1958). It is theoretically possible that alloantibody plays no part in the rejection of allografts, but indirect evidence suggests that antibody may regulate cell mediated immunity. For example:

- (1) The identification of multiple classes of circulating antibodies with distinctive biological properties (Fahey et al., 1964a, 1964b; Adler, 1966; Cohen & Milstein, 1967; Porter & Lieberman,

1967) focuses on the possibility that some classes of antibody do partake in allograft immunity and that this may not be detected using whole hyperimmune anti-sera.

- (ii) Lymphocyte mediated cytotoxicity in vitro is complex and may involve a number of pathways. There is evidence that T cells can be effectors and that humoral antibody can induce cytotoxicity of thymus independent cells (Perlmann et al., 1972) or it can inhibit cytotoxic reactions (MacLennan, 1972). If the conclusions from in vitro work on cytotoxicity can be extrapolated to include cytotoxic reactions that occur during allograft rejection or contact sensitisation, it is possible to envisage a system controlled or partly controlled by different classes or sub-classes of antibody.

It is well established that low molecular weight compounds that elicit contact sensitisation elicit hapten specific antibodies (Benacerraff & Gell, 1969). The role of these antibodies in initiating or regulating the extent of the lesions associated with contact sensitisation is equivocal. Circulating antibodies have been detected in the serum of mice sensitised with oxazolone (Askenase & Asherson, 1972) and evidence suggested that  $\gamma G2$ , which is cytophilic for macrophages, may be responsible for the ability of macrophages to transfer contact sensitisation to oxazolone passively (Zembala & Asherson, 1970; Askenase, 1971). This information accords well with the reports which suggest that antibody cytophilic for macrophages enhances cell-mediated immunity, which has been induced by certain antigens (Parish, 1972). In the present

studies various aspects of the antibody response to oxazolone were investigated:

- (1) The primary antibody response over a period of 42 days was followed. Some mice were given a second application of oxazolone 21 days after the first, and the antibodies produced as a result of the second contact were assayed. The secondary antibody response to particulate antigens such as sheep red cells is well defined (Celada, 1971) and provides a reference point for comparing the 'secondary' response to oxazolone.
- (11) Class specific-antisera were used to detect antibodies of the classes  $\gamma G1$ ,  $\gamma G2a$ ,  $\gamma G2b$  and  $\gamma A$  in the sera of mice undergoing a primary and secondary response.

In Chapter II evidence was presented to suggest that there may be some homeostatic mechanism which regulates the T cell proliferative response to oxazolone. In normal mice it is possible that antibody of other serum factors may regulate, that is suppress, the response to oxazolone. There is no direct evidence to support this suggestion, but there is evidence from a variety of sources that in certain experimental systems, both antibody and other unspecified serum factors may be responsible for inhibiting T cell mediated immunity. For example, it is well established that antibody can suppress or enhance lymphocyte cytotoxicity in in vitro systems (Perlmann et al., 1972; MacLennan, 1972). It has been suggested that antibody may modulate the extent of the histological lesions associated with some types of delayed hypersensitivity (Turk et al., 1972; Turk & Parker, 1973). Recent experiments have demonstrated how important is the route and dose of

antigen in determining the outcome of an immune response, that is the contribution made by circulating humoral antibody and that made by T cells effecting delayed hypersensitivity (Lagrange et al., 1974a). These and other experiments suggested that sensitised effector T cells could be inhibited from expressing delayed hypersensitivity to sheep erythrocytes by antibody to sheep erythrocytes (Lagrange et al., 1974b; Mackaness et al., 1974). The suggestion that some classes of antibody can inhibit some T cell-mediated immunity, is consistent with the idea of an inverse relationship between delayed hypersensitivity and humoral antibody production, a suggestion supported by Parish and co-workers (1972).

Less convincing and sometimes confusing evidence has suggested the existence of factors in immune serum (Monzoian et al., 1974) or in calf thymus (Kiger et al., 1972) or calf spleen (Garcia-Giralt et al., 1972) that can non-specifically suppress T cell-mediated immunity, and in some instances T cell proliferation. The array of factors identified have not, as yet, been characterised sufficiently to be directly comparable. It is possible that some instances of suppression could involve a variety of factors acting in different ways. Some may include inhibitors of a non-immunological nature such as the tissue specific mitotic inhibitors, "chalones" (Bullough & Rytomaa, 1966). Despite the equivocal nature of some of this evidence, it would appear to justify the investigation into whether passively administered hyperimmune serum to oxazolone could suppress the proliferative response in normal animals.

## MATERIALS AND METHODS

### Mice

3-4 month old male mice of the inbred strain CBA/H were used.

### Immunisation of mice

Mice were sensitised with 10 mg of oxazolone dissolved in 0.1 ml of absolute alcohol at 60°C. They were shaved on one side of the thorax and the oxazolone was applied with a pipette. Control mice were shaved and received 0.1 ml of hot alcohol.

### Preparation of sera

Mice were bled from the retroorbital plexus. The blood was allowed to clot at room temperature, the serum removed and stored at -60°C.

### Measurement of DNA synthesis

$^{125}\text{I}$ -labelled 5-iodo-2'-deoxyuridine ( $^{125}\text{IUdR}$ ) was obtained from the Radiochemical Centre, Amersham, and was adjusted immediately before use to a specific activity of 5 mCi/mg by the addition of cold IUdR. Mice received  $5 \times 10^{-8}$  moles of 5-fluorodeoxyuridine (FUdR, Roche Products, Ltd.) in 0.1 ml intraperitoneally one hour before receiving 1  $\mu\text{Ci}$  of  $^{125}\text{I}$ -UdR in 0.1 ml by the same route (Hughes *et al.*, 1964; Pritchard & Mickleth, 1972). The mice were killed two hours later and the axillary and brachial lymph nodes and spleen were removed and fixed in 4% formaldehyde and alcohol. The tissues were washed in 70% alcohol for several days to remove unincorporated  $^{125}\text{I}$ -UdR

(Elkins, 1970). The axillary and brachial nodes from one side of the animal were counted together for 5 minutes, the spleens were counted separately for one minute, and the injected dose was counted in an Auto-Packard Gamma counter. The background counts were subtracted from the organ counts, and the counts per minute were adjusted to the counts that would have been obtained from an injected dose of  $10^6$  cpm. The calculation was therefore as follows:

$$^{125}\text{I-UdR uptake} = \log_{10} \left[ \frac{\text{counts from organ in cpm} \times 10^6}{\text{injected dose in cpm}} \right]$$

It can be seen that the terms 'cpm' cancel out, and the  $^{125}\text{I-UdR uptake}$  is the  $\log_{10}$  of the proportion of the injected dose that was incorporated.

#### Detection of antibodies to oxazolone

The method of coupling sheep red cells (sheep cells in Alsevers', Tissue Culture Services Ltd., Slough) to oxazolone and the haemagglutination technique were performed essentially as described by Askem and Asherson (1972).

##### 1. Coupling of sheep erythrocytes to oxazolone

**Buffers:** the buffers which were used were phosphate buffered saline (PBS), pH 7.4; Ethylenediaminetetraacetic acid, disodium salt (EDTA), pH 8.4; and EDTA-saline, pH 7.5.



Procedure: 0.25 mls of 1% oxazolone in ethanol (10 mg/ml) was squirted using a 1 ml syringe and a 27 gauge needle, into 10 mls of EDTA, pH 8.4. This was immediately added to 5 mls of 30% washed sheep erythrocytes in PBS. It is important that the oxazolone/EDTA mixture is added as soon as possible to the blood, otherwise a precipitate forms and coupling is unsuccessful. The oxazolone, EDTA and blood mixture was agitated gently at room temperature for 30 mins. The reaction was stopped by adding 4 vols of cold EDTA-saline, pH 7.5. The cells were washed 3 times in PBS. Some lysis of the red cells occurred during this procedure.

The coupled cells were stored as a 5% suspension in PBS at 4°C and were used either the same day or the next day.

## 2. Direct haemagglutination technique

A 1:4 dilution of mouse serum which had been previously absorbed with sheep erythrocytes was serially diluted two fold (using Takatsky loops) in 0.05 mls of PBS containing 2.5% normal rabbit serum in 'V' bottom microtitre plates (Biocult Linbro ls MCV 96). The contribution made by 19S (2-mercaptoethanol-sensitive) and 7S (2-mercaptoethanol-resistant) antibodies was estimated. Each serum sample was diluted in duplicate rows of wells. 0.05 mls of 0.2 M 2-mercaptoethanol was added to each well of one row, the control wells received 0.05 mls of diluent. 0.05 mls of 0.1% oxazolone-coupled red cells in diluent was then added to all wells. The plates were covered with a Microtitre plate sealer (Cooke Engineering Co.) and incubated at room temperature for 1-2 hours, and at 4°C overnight before being read. The last well showing

agglutination was taken as the end point.

3. Haemagglutination technique to detect class specific immunoglobulin

Rabbit antisera against mouse immunoglobulins,  $\gamma$ G1,  $\gamma$ G2a,  $\gamma$ G2b,  $\gamma$ A and total  $\gamma$ G (nomenclature after Herzenberg et al., 1969) were prepared by Josephine Riddaway as described by Pritchard et al., (1973).

The following assay was kindly performed by Janice Ure. The direct haemagglutination technique was performed as described above. The plates were incubated overnight and the titres of total antibody and ME-resistant antibody were read. The supernatant was then removed without disturbing the pellet and 0.1 ml of dilute anti-serum added to the wells that had received mercaptoethanol. The contents of the wells were resuspended, recovered and incubated overnight at 4°C. The last well to show agglutination was read as the end point. Pilot studies by Janice Ure showed the optimal dilution of rabbit anti-sera. Thus anti- $\gamma$ G was used at 1:800, anti- $\gamma$ G2b at 1:400, anti- $\gamma$ A at 1:400. Control sera were of two types:

- (i) sera from immunised mice was tested against uncoupled red cells,
- (ii) sera from unimmunised mice were tested against oxazolone-coupled red cells.

The results are expressed as  $\log_2$  of the titre. Thus if the greatest dilution to show agglutination was 1:4, this was expressed at  $2^2$ .

## RESULTS

### 1. Antibodies in the sera of mice given one or two applications of oxazolone

Mice were painted with 10  $\mu$ g of oxazolone distributed between two shaved areas, one on each side of the flank. Half the mice were painted with a second application of 10  $\mu$ g of oxazolone on the same shaved regions, 21 days after the first application. Individual mice were not bled more frequently than once every three weeks. The sera from these mice were stored at  $-60^{\circ}\text{C}$  and used for the two experiments which are represented in Figs. III.1-III.6.

In Figs. III.1 and III.2, antibodies were detected by direct haemagglutination. In Figs. III.3 and III.4 the same serum samples as used above were augmented with anti- $\gamma$  G sera.

In a separate experiment, Figs. III.5 and III.6, the resistant antibodies were augmented with class specific anti-mouse immunoglobulin sera.

Each point on a graph represents the mean titre of the sera from 5 mice.

#### a) Undeveloped antibody, Figs. III.1 and III.2

The primary response over 40 days is shown in Fig. III.1. Sera from unimmunised mice gave titres of  $2^2$  or less. No antibody above that found in unimmunised mice was found on days 3, 4 or 5. On day 6 however, antibody titres, including both  $\gamma$  M and 2-ME resistant antibody were found at titres of  $2^6$ - $2^7$ ; the 2-ME resistant antibody appeared to be declining by day 28 and was no longer detectable by

day 40. The titres of 2-ME sensitive antibodies remained constant between days 14 and 40.

The response to a second administration of oxazolone was measured 3,7,14,20,35 and 42 days after the second dose. Antibody of titres  $2^7$ - $2^8$  were found on day 3. This corresponds to the amount of antibody which was present in the sera before the administration of the second dose. Between days 7 and 14, however, there was a boost to the response, measured as an increase in the titre of both the total and the 2-ME resistant fractions of antibodies. The total response appeared to decline between days 14 and 21 to a level of  $2^8$  which remained constant over the time period studied. The response thus showed higher titres of both  $\gamma$ M and 2-ME resistant antibody over the whole period, than was found during the primary response (Fig. III.1) and there was no decline in the levels of 2-ME resistant antibody.

b) Developed antibody, Figs. III.3 and III.4

The total antibody and the 2-ME resistant antibody were augmented with anti- $\gamma$ G sera, and the results shown in Figs. III.3 and III.4. The primary response is shown in Fig. III.3. It can be seen that the anti-sera developed antibodies on day 5 that had not been revealed using direct haemagglutination (Fig. III.1). Titres of  $\gamma$ G reached  $2^{10}$ - $2^{11}$  on day 10 (possibly before) and remained at this level over the time period which was studied. Augmentation with anti-sera did not reveal a fall in the  $\gamma$ G on day 21 as the previous results in Fig. III.1 had suggested.

The secondary response is shown in Fig. III.4. The pattern of the response is similar to the undeveloped results (Fig. III.2), but are all several wells higher. Unfortunately, the titres which were present on days 7 and 14 were above  $2^{15}$  and were not repeated to obtain the exact end-point.

c) Augmentation of antibodies with anti- $\gamma$ G1, anti- $\gamma$ G2a, anti- $\gamma$ G2b, and anti- $\gamma$ A. Figs. III.5 and III.6

Fig. III.5 shows the results of augmenting the 2ME-resistant antibody produced from mice which had been given a single application of oxazolone, with anti-sera against different classes of antibody. Sera from unimmunised mice gave titres below  $2^2$  in both direct and developed assays. The total  $\gamma$  G of the primary response appeared to consist predominantly of  $\gamma$ G1. Some  $\gamma$ G2a and  $\gamma$ G2b were detected but not until days 21 and 35. The day 10 results for  $\gamma$ G2a and  $\gamma$ G2b showed one mouse out of 5 producing these classes of antibody. No augmentation was found with anti- $\gamma$ A sera.

Fig. III.6 shows the results of developing the 2ME-resistant antibody from mice which had received 2 applications of oxazolone. Anti- $\gamma$ G1 and anti- $\gamma$ G2a augmented the response almost to the same degree, although the day 5 response in  $\gamma$ G1 was too high to be measured exactly. There was augmentation by anti- $\gamma$ G2b serum. No development was produced with anti- $\gamma$ A serum.

2. The effect of passively administered antibody on the proliferative response of lymph node cells to oxazolone. Table III.1

Hyperimmune serum was obtained from mice 14 days after their third

exposure to oxazolone. Normal (unirradiated) mice were injected intravenously with hyperimmune serum or saline, followed one hour later by a skin application of oxazolone or alcohol. The following groups of mice were thus obtained:

Group A received 0.125 mls of hyperimmune serum and oxazolone

Group B received 0.125 mls of saline and oxazolone

Group C received 0.125 mls of hyperimmune serum and alcohol

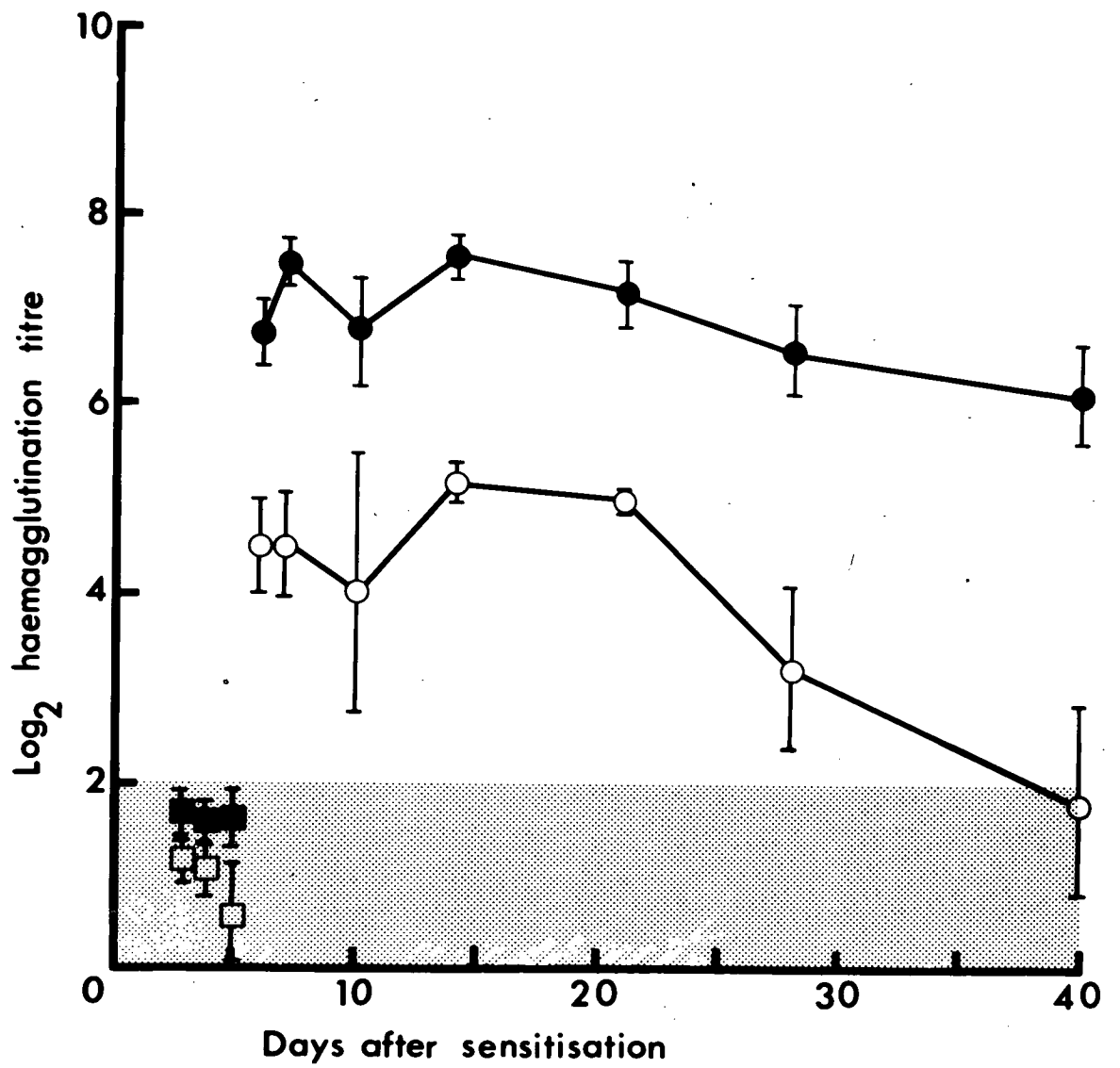
Group D received 0.125 mls of saline and alcohol

The animals were injected with  $^{125}\text{I}$ -UdR 3 and 6 days after they had received oxazolone or alcohol. The proliferative response in their draining axillary and brachial lymph nodes, and spleen were estimated by measuring the amount of  $^{125}\text{I}$ -UdR that was incorporated. The response was measured 3 days after oxazolone in order to detect mainly T cells and 6 days after oxazolone to detect mainly B cells (Davies et al., 1969b). As can be seen from Table III.1, there was no significant difference in the amount of DNA synthesised by mice which had received serum and mice which had received saline, on either days 3 or 6.

**Fig. III.1**

**Titres of haemagglutinating antibody in sera of CBA mice after receiving a single application of 10mg of oxazolone before addition of developing antiglobulin serum. Each point represents the mean titre,  $\pm$  SE.**

**○, 2-ME resistant; ●, total; shaded area represents titres produced by sera from unimmunised mice.**





**Fig. III.2**

**Titres of haemagglutinating antibody in sera of CBA mice after receiving two applications of 10mg of oxazolone before addition of developing antiglobulin serum. Each point represents the mean titre,  $\pm$  SE. ①, 2-ME resistant; ● , total; shaded area represents titres produced by sera from unimmunised mice, □ , 2-ME resistant, ■ total.**

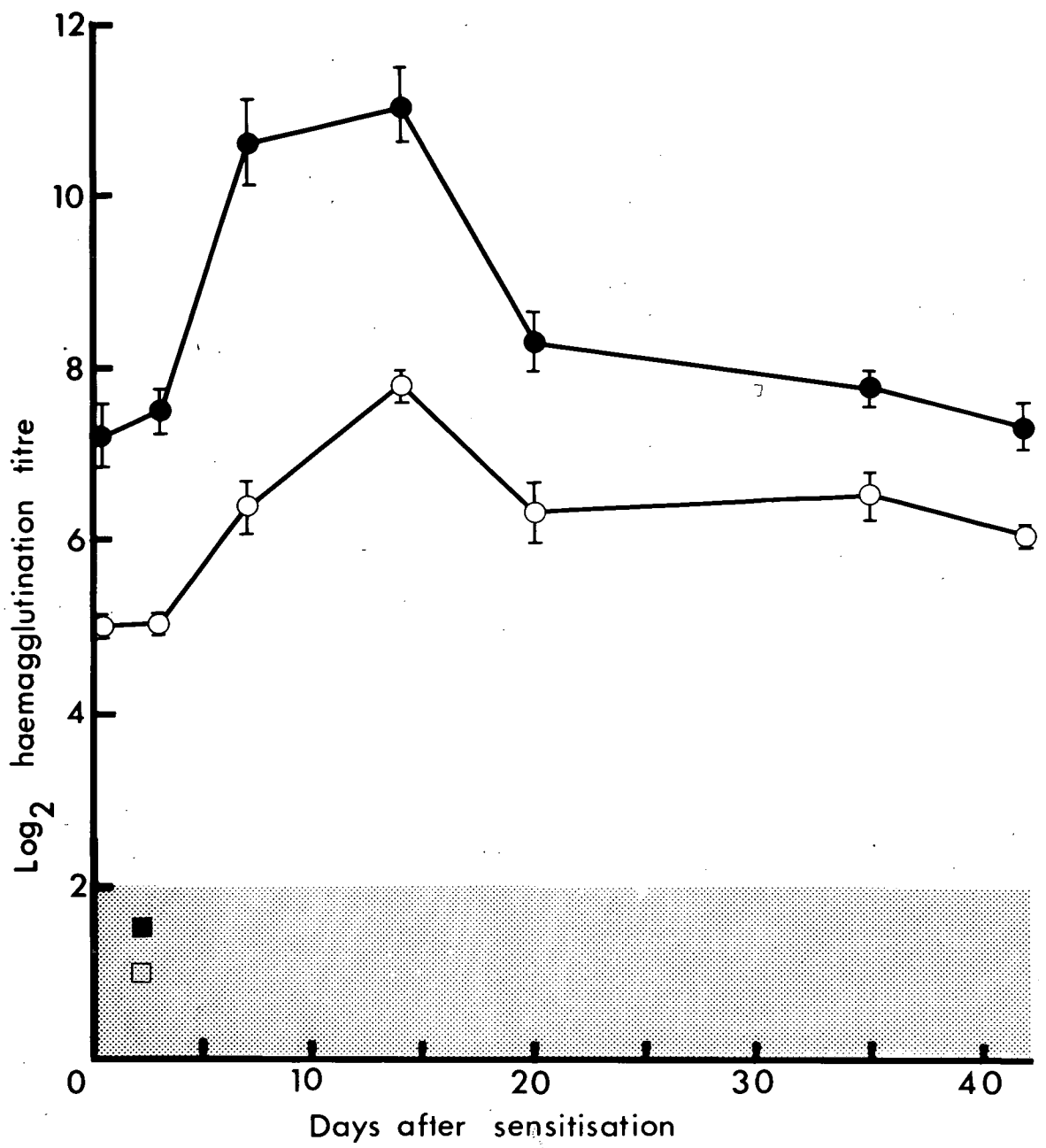
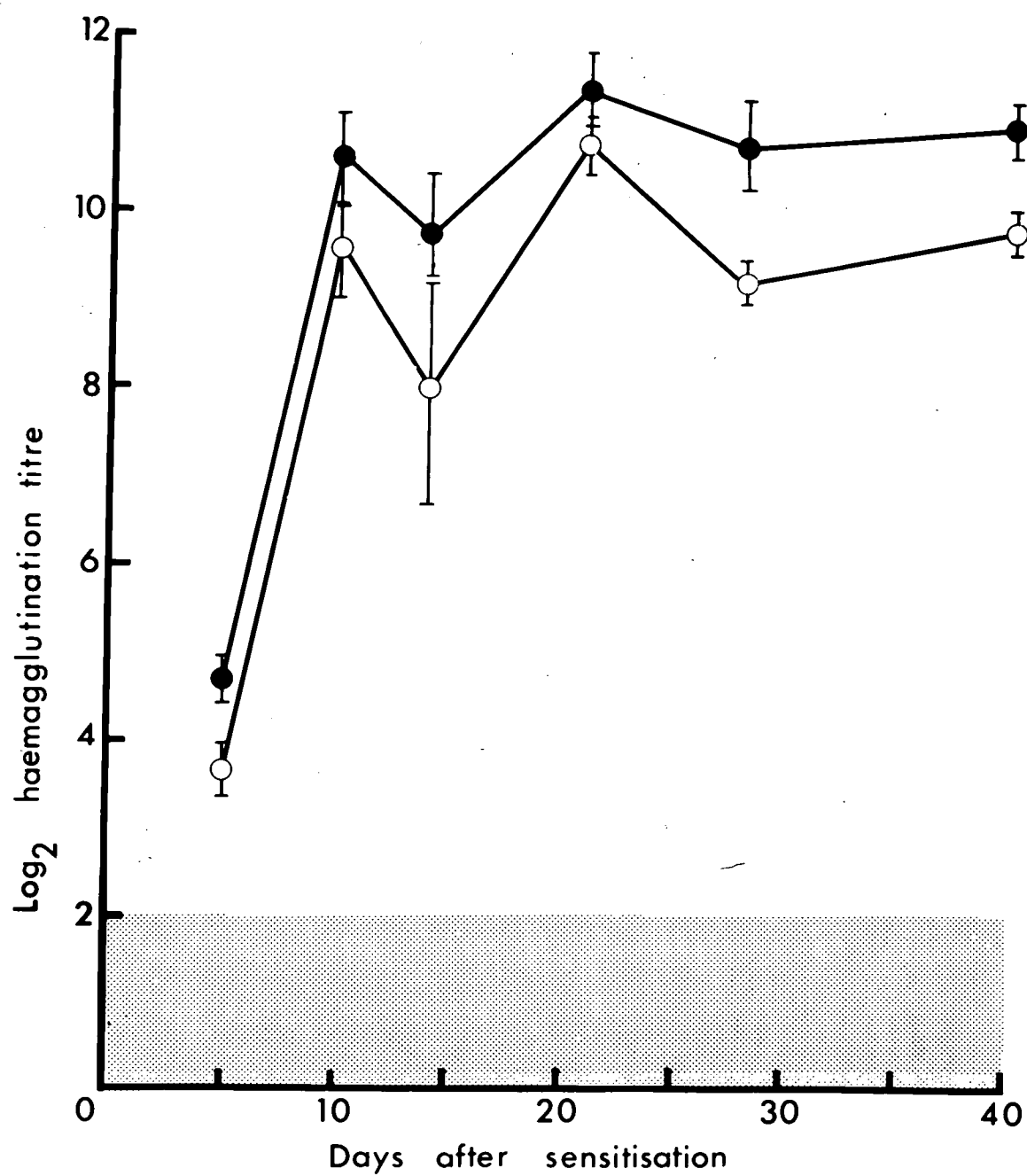


Fig. III.3.

Titres of haemagglutinating antibody in sera of CBA mice after receiving a single application of 10mg of oxazolone, after the addition of developing antiglobulin serum. Each point represents the mean titre,  $\pm$  SE. ①, 2-ME resistant; ●; total; shaded area represents titres produced by sera from unimmunised mice.



**Fig. III.4**

**Titres of haemagglutinating antibody in sera of CBA mice, after receiving 2 applications of 10mg of oxazolone, after addition of developing antiglobulin serum. Each point represents the mean titre,  $\pm$  SE. ①, 2-ME resistant; ●, total; shaded area represents titres obtained from sera of unimmunised mice.**

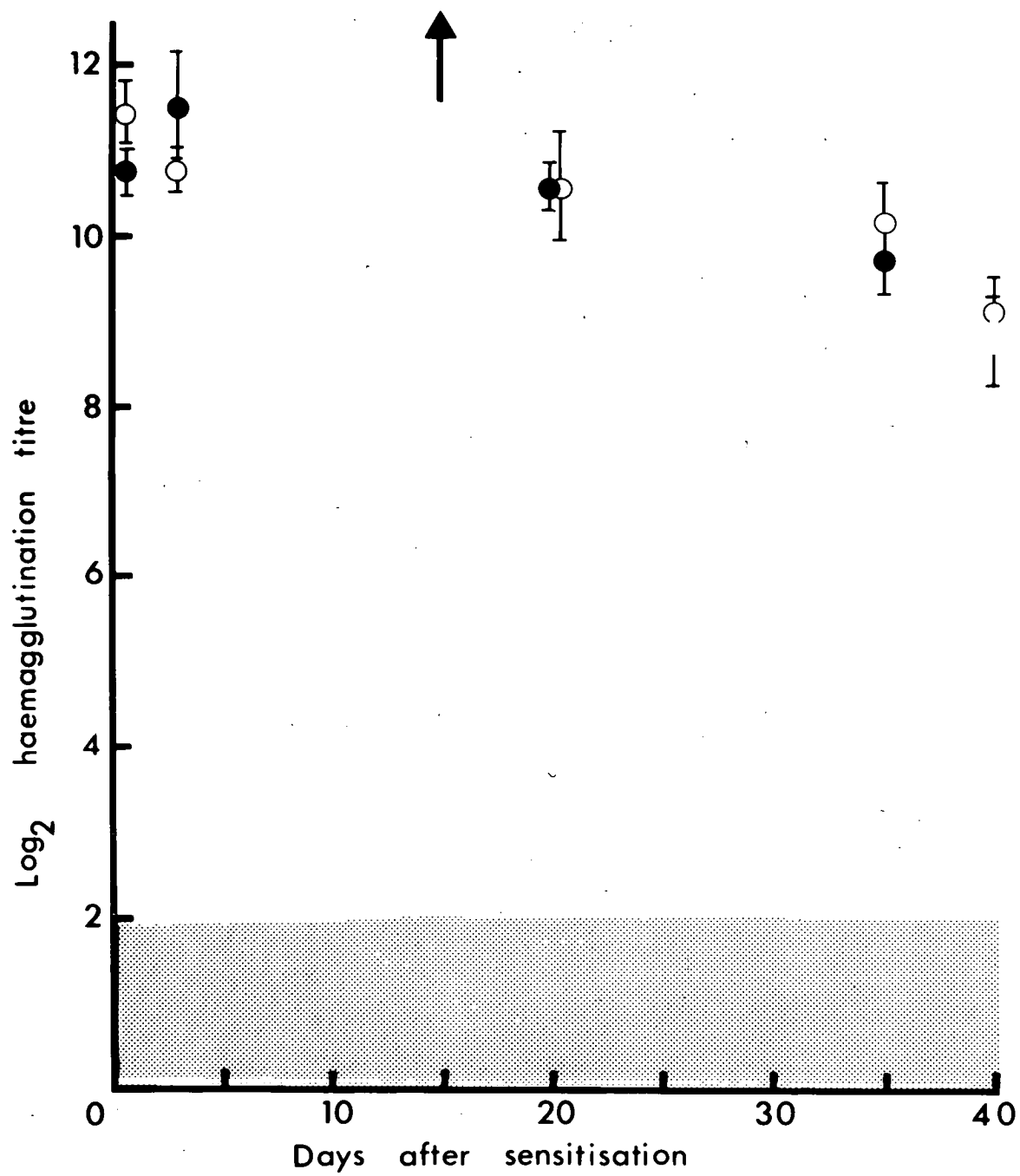


Fig. III.5.

Augmentation of 2 ME-resistant haemagglutinating antibody in sera of CBA mice, after receiving a single application of oxazolone, with anti- $\gamma$ G serum; anti- $\gamma$ G<sub>1</sub> serum; anti- $\gamma$ G<sub>2a</sub> serum; anti- $\gamma$ G<sub>2b</sub> serum; anti- $\gamma$ A serum. "Undeveloped antibody" represents haemagglutinating antibody in the absence of mercaptoethanol and developing anti-sera. The shaded areas represent 2-ME resistant antibody before addition of developing anti-serum. The dotted lines show titres obtained from the sera of unimmunised mice.

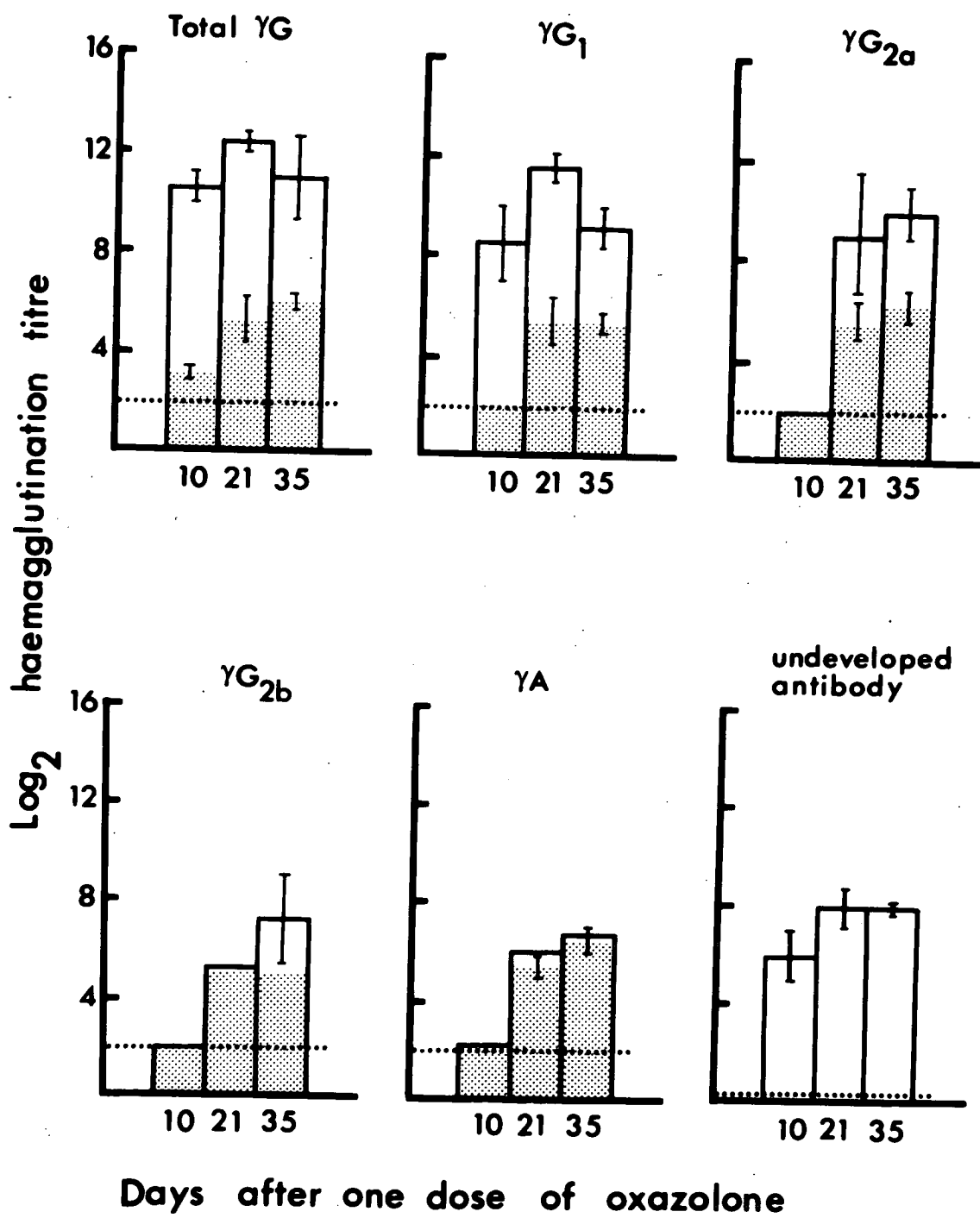




Fig. III.6

Augmentation of 2ME-resistant haemagglutinating antibody in sera of CBA mice, after receiving two applications of oxazolone, with anti- $\gamma$ G serum; anti- $\gamma$ G<sub>1</sub> serum; anti- $\gamma$ G<sub>2a</sub> serum; anti- $\gamma$ G<sub>2b</sub> serum; anti- $\gamma$ A serum. 'Undeveloped antibody' represents haemagglutinating antibody in the absence of mercaptoethanol and developing anti-serum. The shaded areas represent 2-ME resistant antibody before addition of developing anti-serum.

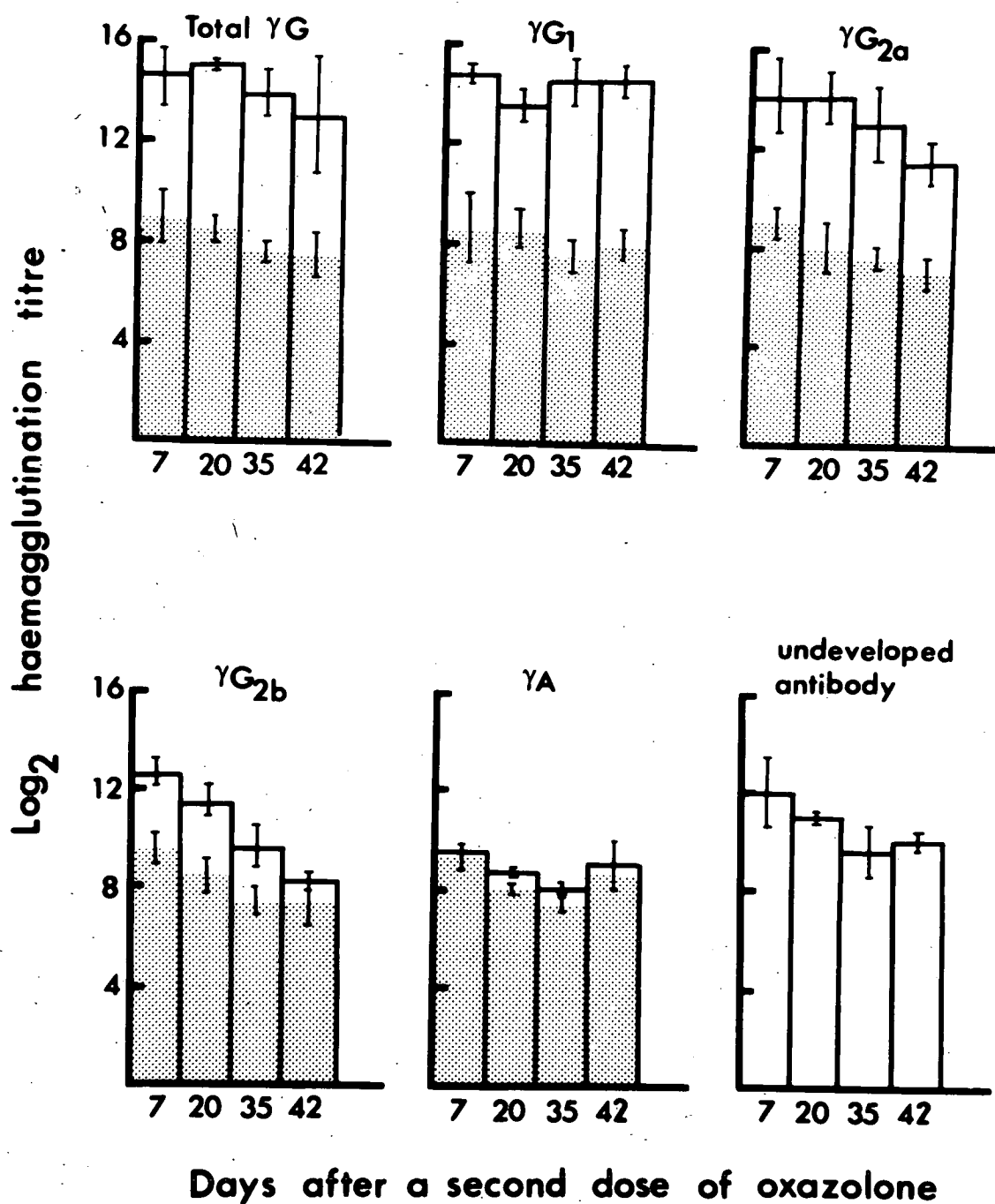


TABLE III.1

Effect of passive antibody on the proliferative response of the  
axillary and brachial lymph nodes and spleen

Group	No. mice	<sup>125</sup> IUdR, mean $\pm$ SE					
		Day 3			Day 6		
		Draining	Contralateral	Spleen	Draining	Contralateral	Spleen
A	4	3.15 $\pm$ 0.006	2.66 $\pm$ 0.12	3.82 $\pm$ 0.02	2.95 $\pm$ 0.03	2.11 $\pm$ 0.14	3.88 $\pm$ 0.03
B	4	3.09 $\pm$ 0.05	2.51 $\pm$ 0.10	3.80 $\pm$ 0.05	2.88 $\pm$ 0.06	2.15 $\pm$ 0.16	3.83 $\pm$ 0.20
C	4	1.82 $\pm$ 0.10	1.65 $\pm$ 0.17	3.00 $\pm$ 0.13	1.85 $\pm$ 0.14	1.90 $\pm$ 0.09	3.63 $\pm$ 0.04
D	4	1.69 $\pm$ 0.16	1.76 $\pm$ 0.06	3.15 $\pm$ 0.09	2.07 $\pm$ 0.05	2.04 $\pm$ 0.05	3.62 $\pm$ 0.07

Group A received hyperimmune serum and oxazolone

" B received saline and oxazolone

" C received hyperimmune serum and alcohol

" D received saline and alcohol

## DISCUSSION

The method of detecting antibodies to oxazolone was essentially the same as that described by Askenase and Asherson (1972). They found that using a constant amount of oxazolone and increasing the numbers of sheep cells to be coupled, they detected less antibodies in the sera of unimmunised mice. In the present studies a mixture of 30% sheep red cells were coupled to oxazolone. Preliminary studies consisted of coupling 15% sheep red cells to oxazolone, but it was found that these coupled red cells detected fairly high titres in unimmunised mice, thus it was decided to use 30% sheep erythrocytes. All titres obtained in these results were lower than the general levels obtained by Askenase and Asherson (1972), possibly because they coupled oxazolone to 15% sheep red cells. Thus in the present studies, the technique may have been detecting a narrow spectrum of antibodies (of high avidity) compared to the study of Askenase and Asherson.

The antibody response to one and two applications of oxazolone was examined to investigate whether there was a distinguished secondary response and to compare the features of this response to those obtained using well documented antigens such as heterologous red cells. The response to a second dose of oxazolone was different from the first response, in that it reached titres of  $2^{11}$  (1:2048) compared to the peak response to the first antigen of  $2^7$  (1:128). The secondary response is thus considerably greater than can be accounted for by the sum of the antibody already present plus the antibody formed by a duplicate response. The studies which were reported in Chapter II (Experiment 5)

suggested the existence of a Factor of Immunisation of oxazolone which was mediated definitely by T cells and possibly by B cells. The function of the T cells may only be concerned with the histological lesions associated with delayed response, but they could also contribute as 'helper' cells to the memory component of the humoral response (Mitchison, 1971).

The characteristics of a secondary humoral response to heterologous red cells that distinguish it from a primary response have been well documented (Celada, 1971). They are as follows:

- (i) a reduced or absent lag period before the appearance of antibody forming cells;
- (ii) an earlier and higher peak of antibody forming cells and circulating antibody, often of higher affinity;
- (iii) an early bias towards the synthesis of 7S antibodies.

Information about the features of the secondary humoral response to antigens that are grafted or painted on the skin is sparse (Celada, 1971).

In the present studies mice were given a second dose of oxazolone 21 days after the first. At this time, as can be seen from Fig. III.1 the primary response had not yet declined. In discussing the results shown in Fig. III.2 it is therefore necessary to distinguish between a boosted primary response and a true secondary response. A secondary response implies cellular differentiation as a result of contact with the second dose of antigen that is qualitatively different from the cellular differentiation that occurs as a result of initial contact between unprimed lymphocytes and antigen. In other words, according

to the X, Y, Z scheme of Sercarz and Coons (1962), in the present studies it is not possible to distinguish whether, as a result of the second application of oxazolone, more X cells are going to Z cells, or whether the Y cells are differentiating to Z cells. Nevertheless, within the limits imposed by this uncertainty it is possible to discuss the features of the secondary response to oxazolone with reference to the criteria described above. The presence of a reduced or absent lag period before the appearance of circulating antibody in the secondary response

(Fig. III.2) cannot be ascertained because it is obscured by the antibody present at the time the second dose was administered. Regarding the second criterion, higher titres of antibody were produced between days 7 and 14 of the secondary response than at any time of the primary response. There appeared to be little difference between the contribution of 7S (2-ME resistant) and 19S to the titres of either the primary or the secondary response. The highest titres of antibody produced during the secondary response occurred between days 7-14, not demonstrably earlier than the peak titres which occurred during the primary response. The third criterion is not appropriate because  $\gamma$ G was detected early during the primary response simultaneously with the detection of  $\gamma$ M. It may be significant that the  $\gamma$ G declined after day 21 during the primary response, although augmentation with anti- $\gamma$ G serum did not confirm this trend. Thus quantitatively the response to a second dose of oxazolone was considerably greater (about twenty-fold between days 7 and 14) than the response to one dose of antigen.

Figs. III.5 and III.6 are the results of experiments which utilised class-specific anti-sera to augment the antibody titres which were

detected during the primary and secondary responses. They suggest that there may be qualitative differences between the responses. In the primary response the major contribution to the  $\gamma$ G was made by  $\gamma$ G1 antibody with some participation by  $\gamma$ G2a and very little  $\gamma$ G2b. In the secondary response, however, there was a considerably greater contribution made by  $\gamma$ G2a and  $\gamma$ G2b. Anti- $\gamma$ A sera failed to detect any  $\gamma$ A antibodies to oxazolone, which accords with the results reported by Askenase and Asherson (1972). This is not particularly surprising in view of what is known about  $\gamma$ A.  $\gamma$ A is manufactured chiefly by the gut associated lymphoid tissue (Mandel & Asofsky, 1968; Guy-Grand et al., 1974) and tends to be lost in various secretions (Tomasì & Zigelbaum, 1963; Tomasì et al., 1965; South et al., 1966). It is interesting that the present studies detected  $\gamma$ G2 antibody in both the primary and secondary responses, in view of the cytophilic nature of  $\gamma$ G2 (Nelson et al., 1967; Hoy & Nelson, 1969) and its possible role in mediating delayed hypersensitivity when attached to macrophages (Zembala & Asherson, 1970; Askenase, 1971; Parish, 1972).

It seems conceivable that there could be qualitative differences in the antibody responses which are initiated by antigens which are painted on the skin and antigens, such as heterologous erythrocytes, which are injected intravenously or intraperitoneally. Such differences could arise from several sources. One is the nature of the antigen and the fact that low molecular weight sensitising agents combine with epidermal amino-groups. This produces antigenic epitopes which may be more

similar to 'self-antigens' than are the epitopes found on heterologous erythrocytes. Secondly is the way the antigen is presented to the immunocompetent cells and the involvement of macrophages. Thus oxazolone may reach the draining lymph nodes in soluble combination with protein in the lymph (Hall & Smith, 1971) which could affect the way it is handled by macrophages, its presentation and persistence in an immunogenic form. These factors are important in determining the nature of the primary and secondary response (Celada, 1971).

In experiment 2 an attempt was made to suppress the proliferative response of lymph node lymphocytes to oxazolone with passively administered antibody. It is well documented that passive antibody can suppress both  $\gamma$ M and the  $\gamma$ G production by B cells in vivo (reviewed by Uhr & Möller, 1968) and in vitro (Lang et al., 1969; Pierce, 1969; Feldmann & Diener, 1970). The way in which passive antibody affects delayed hypersensitivity is however equivocal. It is well known that passive antibody directed against allografts may suppress the rejection process, a phenomenon known as "immunological enhancement" (Kaliss, 1958; Snell et al., 1960; Möller & Möller, 1966; Uhr & Möller, 1968). The manifestation of delayed hypersensitivity produced by antigen other than allografts appears to be less easily suppressed by passive antibody (Uhr & Möller, 1968) and indeed it has been shown that passive antibody can promote the delayed response (Liew & Parish, 1972). There is some evidence in the literature that the delayed response to sheep erythrocytes can be suppressed or diminished by passive antibody to sheep red cells (Axelrad, 1968; Mackaness et al., 1974), and Crowle and Hui (1965, 1968) suppressed the delayed, but not the immediate,



hypersensitivity response to ovalbumin with passive antibody to ovalbumin.

Indirect evidence that antibody may regulate the extent of the histological lesion associated with some types of delayed hypersensitivity stems from the experiments of Turk et al. (1972, 1973). They found that pretreating guinea-pigs with cyclophosphamide 3 days before sensitisation with dinitrofluorobenzene (DNFB) results in an increase in the intensity and duration of the contact sensitisation response which is elicited by challenge with DNFB. The results of experiment 2 failed to detect any effect of anti-sera against oxazolone on the proliferative response of lymph node cells to oxazolone. It was reasoned that the anti-sera could act by either combining with antigen and thus inhibiting the recognition of antigen by antigen sensitive T cells, or by acting directly on antigen sensitive lymphocytes. The antibody was administered on the same day as the oxazolone as has been shown to be necessary to elicit a suppressive response with other antigens (Lang et al., 1969; Pierce, 1969; Möller & Wigzell, 1965). A criticism of the experiment is that only one injection of hyperimmune serum was administered and previous workers who achieved suppression used anti-sera daily for 7 days (Crowle & Hu, 1965, 1968) or for 3 days (Axelrad, 1968).

Some corroboration of these results is provided by Turk et al. (1972), who did not detect an increase in paracortical proliferation in the lymph nodes of cyclophosphamide treated guinea-pigs, and Mackaness et al. (1974) who suppressed the delayed hypersensitivity response to sheep erythrocytes by passively administered IM without suppressing the

uptake of  $^3\text{H}$ -thymidine in the draining lymph nodes.

There is little information in the literature regarding the effect of antibody on T cell proliferation. There is however evidence from a variety of sources that a factor can be isolated from immune sera or lymphoid organs that can suppress, non-specifically, T cell mediated immunity, an example of such a factor is "Immunoregulatory Alphaglobulin". The relationship between 'Immunoregulatory Alphaglobulin' and the other T cell suppressive factors that have been reported (Howbray, 1963; Carpenter et al., 1971; Garcia-Giralt et al., 1972; Kiger et al., 1972; Ambrose, 1973) is unknown. These reports include several examples where the factor has been postulated as preventing T cell activation and proliferation (Cooperband et al., 1972; Carpenter et al., 1971; Garcia-Giralt et al., 1972). It is conceivable that some of the factors involved in suppressing T cell proliferation in the above examples are tissue-specific mitotic inhibitors such as 'chalones' (Bullough & Rytomaa, 1965). Evidently some mechanism regulates and suppresses T cell proliferation, the nature of the mechanism, whether it is a class of antibody or a factor released by T cells, or indeed other cells remains unknown.

SUMMARY

1. Antibody of classes  $\gamma$ G1,  $\gamma$ G2a,  $\gamma$ G2b and  $\gamma$ M against oxazolone, were detected in the sera of mice which had been immunised with oxazolone, confirming the results of Askenase and Asherson (1972).
2. The response to a second dose of oxazolone was distinguishable from the response to one dose by the following features:
  - a) the titres of antibody produced in the secondary response were greater than those produced during the primary response.
  - b) there were more antibodies of the class  $\gamma$ G2 produced during the secondary response.
3. No experimental evidence was obtained to suggest that the mechanism which regulates the proliferation of lymphocytes to oxazolone includes factors in hyperimmune serum.

CHAPTERS IV & VCELL RECRUITMENT DURING THE RESPONSE TO OXAZOLONE

The important phenomenon that lymphoid tissues respond to antigen primarily by means of the cells circulating through them, as opposed to residential cells, was demonstrated by experiments that showed heavily irradiated lymph node (Hall & Morris, 1964) or spleen (Taliaferro & Taliaferro, 1956; Ford & Gowans, 1967) were capable of giving normal haemolysin responses, provided they were perfused with lymphocyte rich medium. The recruitment of circulating cells by antigen stimulated lymphoid tissue is therefore an early event in the initiation of the immune response. It follows that an understanding of the kinetics of cell circulation, the factors that govern it and the mechanism of cell recruitment must provide insight into the initiation and possibly regulation of the immune response.

Chapter IV describes the development of an isotopic label, hitherto unused to study cell localisation. Chapter V is a study of the kinetics of cell recruitment during the response to oxazolone. The following account briefly describes our present understanding of lymphocyte circulation and recruitment.

## INTRODUCTION

### 1. Lymphocyte Circulation

#### 1a. The kinetics of T and B cell circulation

Early experiments (1936-1965) which involved the measurement of lymphocyte output from the thoracic duct of various animals revealed that the number of cells entering the blood each day was in excess of the number present in the blood (reviewed by Gowans, 1966). A possible explanation for this phenomenon was the existence of a continuous recirculation of cells between the blood and the lymph. The demonstration that the profound fall in output of lymphocytes which occurs during the chronic drainage from a thoracic duct cannula (Mann & Higgins, 1950) could be prevented in rats by the continual infusion of small lymphocytes (Gowans, 1967) and that thoracic duct cells were not newly formed (Gowans, 1957) supported this contention. The direct evidence which demonstrated blood to lymph circulation was the infusion of  $^{32}\text{P}$  labelled (Gowans, 1957) or  $^{51}\text{Cr}$  labelled (Shorter & Bollman, 1960) lymphocytes intravenously into syngeneic rats and the subsequent recovery of large number of labelled cells in the thoracic duct. Additional support, this time in sheep, was provided by Hall & Morris (1964) who showed that the cells present in the efferent lymphatics of a peripheral lymph node were not newly formed by the lymph node.

The major route of lymphocytes from blood to lymph is through the lymph nodes (Gowans & Knight, 1964). The main point of entry is through the post-capillary venules (Gowans & Knight, 1964) and the main

exists are the efferent lymphatics (Hall & Morris, 1962, 1963, 1964, 1965). Recirculating lymphocytes also pass through the spleen, entering and probably leaving by vascular channels. Evidence for this route of circulation was provided by experiments which involved the intravenous injection of labelled lymphocytes which migrated to the spleen (Gowans & Knight, 1964; Goldschneider & McGregor, 1968a; Parrott & de Sousa, 1971) and the fact that spleens became depleted of cells after thoracic duct drainage (McGregor & Gowans, 1963; Goldschneider & McGregor, 1968a; Sprent, 1973) or extracorporeal irradiation of the blood in the calf (Cottier et al., 1964).

In young adult rats the output of cells from the thoracic duct falls precipitously over three days of drainage until a total of about  $2 \times 10^9$  cells have been withdrawn; after five days the output levels off at a value approximately 10-20% of that on the first day (Gowans, 1957; Caffrey et al., 1962; McGregor & Gowans, 1963). A similar pattern of cell depletion is found during thoracic duct drainage of mice (Sprent, 1973). This pattern of mobilisation suggests the presence of two populations of cells, a rapidly and a slowly recirculating population. It is conceivable that the cells obtained after 48 hours of drainage may not be a normal recirculating pool and may only be obtained as a result of the abnormal stress imposed by chronic cell depletion (Gowans & Knight, 1964).

Thoracic duct lymphocytes consist of about 80% T cells (Miller & Sprent, 1971). In order to study the circulation of the T cell component advantage was taken of the fact that T cells from rats

incorporate about fifteen times as much  $^3\text{H}$ -uridine as B cells (Howard et al., 1972). Ford and Simmonds (1972) studied the time taken for  $^3\text{H}$ -uridine labelled thoracic duct cells to travel from blood to lymph in splenectomised rats. They found a transit time across lymph nodes of 12-18 hours as opposed to a spleen transit period of 2-6 hours (Ford, 1969; Bradfield & Born, 1973). Ford (1969) calculated the size of the intrasplenic pool of recirculating small lymphocytes (in the rat) to be probably  $400-500 \times 10^6$  cells. This pool belongs to the same pool of cells that recirculate through the lymph nodes (Ford, 1968; Bradfield & Born, 1973) and is probably mainly T cells (Sprent, 1973).

Evidence that the B cells present in thoracic duct lymph are recirculating cells stems from studies on the thoracic duct lymphocytes of 'B' rats (Howard, 1972), 'B' mice and athymic nu/nu mice (Sprent, 1973). Howard (1972) showed the thoracic duct lymph of 'B' rats contained 20% as many lymphocytes as normal thoracic duct lymph, and of a similar long lived nature. There are differences in patterns of recirculation between T and B lymphocytes. Sprent (1973) compared the distribution of  $^{51}\text{Cr}$  labelled normal thoracic duct lymphocytes (TDL) and TDL from 'B' mice or nu/nu mice in syngeneic recipients. Similar quantities of B TDL and normal TDL localised in the spleen four hours after intravenous injection, but more normal TDL were found in the lymph nodes. The movement of cells from spleen to lymph nodes, between four and twenty-four hours that characteristically occurs with normal TDL and lymph node cells (Zatz and Lance, 1970) was absent using 'B' TDL. This result may suggest a propensity for B cells to

circulate through the spleen at a slower rate than T cells, and this suggestion is supported by experiments which have shown it takes longer to mobilise B cells by thoracic duct drainage than T cells. After the intravenous injection of labelled rat TDL into syngeneic recipients the peak recovery of cells issuing from the thoracic duct occurred after about eighteen hours of drainage, with low levels of recovery occurring after 24-36 hours (Ford & Simmonds, 1972). Howard (1972), however, discovered that during thoracic duct drainage of 'B' rats, low numbers of cells were recovered after 18 hours compared to normal rats but there was a steady flow of cells between 18-48 hours. The possibility that the later phase of recovery represented B cell mobilisation was also suggested by the finding that the ratio of B/T cells in the thoracic duct lymph of mice increased from 15% after 12 hours of drainage to 50% after four days (Sprent, 1973). Continuous drainage of lymphocytes for three days was sufficient to produce depletion of T areas of spleen but in Nu/Nu mice no depletion of B areas was found after the same treatment (Sprent, 1973).

There is evidence that many of the circulating B cells are memory cells and that unprimed B cells are newly formed sessile lymphocytes (Strober, 1972; Strober & Dilley, 1973a, 1973b).

#### 1b. Circulation within the lymph nodes and spleen

It is well established that within the secondary lymphoid tissue there are discrete compartments to which T and B cells preferentially migrate and occupy (Parrott & de Sousa, 1971; de Sousa, 1973). Lymphoid tissue has been termed ecotaxis (de Sousa, 1971) and elucidating the



mechanisms which control it may contribute to the understanding of how antigen and adjuvants affect cell traffic through lymphoid tissue. Experiments of two types established the existence of T dependent areas within the peripheral lymphoid tissue:

- (i) Histological examination of the lymph nodes and spleen from neonatally thymectomised rats (Waksman et al., 1962) and mice (Parrott et al., 1966), nu/nu mice (de Sousa et al., 1969; de Sousa & Pritchard, 1974), adult thymectomised, bone marrow reconstituted mice (Davies et al., 1969a, 1969b, 1970), and children with di George's syndrome (Cleveland et al., 1968; August, et al., 1970) revealed a paucity of cells within the paracortex and the post-capillary venules of the lymph nodes and the periarteriolar sheath of the splenic Malpighian body.
- (ii) Isotopically labelled cell populations from various sources migrated to distinct areas; labelled thoracic duct cells and thymocytes migrated predominantly to T dependent areas (Gowans & Knight, 1964; Parrott et al., 1966; Parrott, 1967; Goldschneider & McGregor, 1968a; Ford, 1969; Parrott & de Sousa, 1969; Bradfield & Born, 1973; Sprent, 1973). Spleen cells tended to spread over various compartments (Parrott et al., 1966; Parrott, 1967; Parrott & de Sousa, 1969; Mitchell, 1972). Lymph node cells migrated primarily to T dependent territory (Balfour et al., 1971) but also to the peripheral nodules of lymph nodes (Austin, 1969). The cells that were found to migrate to T independent areas were bone marrow cells (Parrott & de Sousa, 1971; de Sousa, 1971), thoracic duct lymphocytes from 'B' mice (Howard, 1972; Sprent, 1973) and nu/nu mice (Sprent, 1973), spleen cells from 'B' mice (Mitchell, 1972) and Ig positive lymph node cells from 'B' mice (Gutman & Weissman, 1973).

These experiments established the T dependent areas to be the paracortex in the lymph nodes and the periarteriolar sheath of the spleen. The areas usually not occupied by T cells are the superficial cortex, germinal centres and medulla of the lymph nodes and the red pulp, germinal centres and surrounding corona in the spleen. T and B cells do, however, share some common territory. Both cell types enter lymph nodes by the post capillary venules (Gutman & Weissman, 1973; Niewenhuys & Ford, 1974). The T cells proceed through the paracortex to the efferent lymphatics, but the B cells move towards the superficial cortex to localise mainly in the germinal centres from where they return to the medulla to enter the efferent lymph. B and T cells also share a common point of entry to the splenic follicles through the sinus in the marginal zone where they show no preference for specific areas (Mitchell, 1972; Gutman & Weissman, 1973; Niewenhuys & Ford, 1974). T cells migrate to the periarteriolar sheath and B cells localise mainly in the lymphatic corona around the germinal centres and in the germinal centres. Bone marrow cells tend to localise in the red pulp (de Sousa, 1971). The routes by which cells leave the spleen are not clear, T lymphocytes possibly leave the periarteriolar sheath to the venous sinus by bridging channels across the marginal zone (Mitchell, 1973). The transit time of B cells through the spleen is thought to be longer than the 6-6 hours time of T cells (Ford, 1975).

Various factors could determine the segregation of cells into these compartments. They include the architecture of the lymphoid organs and properties of the lymphocyte membrane. Examination of the reticulin framework of the lymphoid organs has not revealed any barriers between

the primary nodule and the rest of the lymph node cortex or between the outer and inner layers of the Malpighian follicle in the spleen (Denz, 1947; de Sousa, 1969). Zonal differences do, however, exist that coincide with the thymus dependent and independent areas. The reticulin fibres in the mid cortex of lymph nodes and the periarteriolar sheath of the spleen are arranged in an open pattern (Denz, 1947; Sainte-Marie & Sin, 1968), whereas the medulla and red pulp of the spleen have a comparatively close network. This raises the possibility that some characteristics of T lymphocytes attracts them to the open network and similarly some property of B lymphocytes may attract and retain them in the close network. A possible mechanism for the latter may lie in the existence of a mesh of dendritic cells with long cytoplasmic processes which is found in T independent areas (White, 1963; Miller & Nossal, 1964) and which possesses intercellular deposits of immunoglobulin (Balfour & Humphrey, 1967). It is known that B cells possess Ig receptors (Raff, 1970), receptors for Fc pieces (Basten et al., 1971) and C'3 (Bianco et al., 1970). It is conceivable that the prominent exposure of these receptors would enable the cells to become entangled in the areas where immunoglobulin and antigen/antibody complexes are deposited. At present there is no experimental evidence to support this contention and indeed an attempt to demonstrate that local antigen/antibody complexes attract primed cells was unsuccessful (Balfour et al., 1971).

#### 1c. Circulation of activated T and B cells

There is evidence that some recently activated lymphocytes, that is lymphoblasts, stop recirculating and extravasate from the circulation to areas such as intestinal mucosa, skin and possibly other non-lymphoid

tissue.

5-10% of rat thoracic duct cells are large lymphocytes (Marchesi and Gowans, 1964). When these cells were labelled with tritiated thymidine and injected into syngeneic recipients, large numbers were found in the lamina propria of the small intestine (Gowans & Knight, 1964; Griscelli et al., 1969; Guy-Grand et al., 1974). These cells contain prominent endoplasmic reticulum and are therefore possibly B cells (Marchesi & Gowans, 1964). The lymphoblasts induced in the thoracic duct by a variety of antigens also homed predominantly to the small intestine (Smith & Hall, 1971; Hall et al., 1972; Halstead & Hall, 1972; Parrott & Ferguson, 1974). The antigens that were used in the above experiments, Bordetella pertussis, Corynebacterium parvum, bovine gammaglobulin, sheep red cells, tumour cells, human influenza vaccine and Nippostrongylus, do not exclude the possibility that some of the lymphocytes were T cells, but evidence suggests that many of the cells found in the intestine were B cells. For example, some of the large lymphocytes in the lamina propria differentiated into plasma cells (Smith & Hall, 1971; Hall et al., 1972) and many contained  $\gamma$ A (Guy-Grand et al., 1974). It is interesting that the majority of immunoglobulin containing TDL contain predominantly  $\gamma$ A, in striking contrast to the  $\gamma$ M and  $\gamma$ G content of spleen and peripheral lymph node cells (Jensenius & Williams, 1974; Guy-Grand et al., 1974). Peyer's patches and the mesenteric lymph nodes are also rich sources of  $\gamma$ A (Guy-Grand et al., 1974; Tomasi & Bienenstock, 1968). The search for a correlation between the  $\gamma$ A producing cells in the gut associated lymphoid tissue (GALT), TDL and the propensity for TDL to

home to the gut has led to the concept of a YA cycle (Guy-Grand et al., 1974; Ford, 1975). After antigenic stimulation, the Peyer's patches release YA producing cells which reach the thoracic duct lymph via the mesenteric nodes and efferent lymphatics. The cells are distributed by the blood and home in large numbers to the lamina propria, and in lesser numbers to the spleen, lymph nodes and probably elsewhere. The GALT may not be the only source of lymphoblasts that home to the intestine. This is suggested by the experiments of Hall et al. (1972) and Halstead & Hall (1972) which demonstrated that stimulating animals in the legs with a variety of antigens produced lymphoblasts in the thoracic duct that homed in large numbers (after 24 hours, 80% of the recoverable radioactivity) to the small intestine. Hall et al. (1972) point out that the commitment of a large proportion of each generation of newly formed activated cells to protect the gut would be immunologically advantageous, for the gut has a large surface area and is continually exposed to foreign antigens such as bacteria and pathogens. The fate of the lymphoid cells that settle in the lamina propria is unknown.

The factors which attract or cause blast cells to migrate out of the intestinal capillaries are unknown. The suggestion that the gut antigen that produced the blast cells directs their homing (Dineen et al., 1968), seems untenable in view of the migration of activated TDL to the gut of unsuckled neonates (Halstead & Hall, 1972) and to foetal gut transplants (Parrott & Ferguson, 1974; Guy-Grand et al., 1974). Large lymphocytes produced in the mesenteric lymph nodes tend to return to mesenteric lymph nodes (Griscoll et al., 1969; Guy-Grand et al., 1974) or the intestine (Dineen et al., 1968;

Guy-Grand et al., 1974) suggesting a possible affinity between  $\gamma$ A producing cells and the intestinal mucosa.

Any conclusions regarding the migration of activated T cells to the gut are tentative at present, but a consensus of opinion from the available evidence would probably be that activated T cells migrate to the gut in smaller amounts than activated B cells. Parrott and Ferguson (1974) found that  $^3\text{H}$ -thymidine labelled lymphoblasts from mesenteric nodes of young mice and mice injected with Nippostrongylus brasiliensis were found in Peyer's patches and the lamina propria, while lymphoblasts from oxazolone primed lymph nodes did not migrate to villi. Ford and Sedgely (reported in Ford, 1975) produced activated T cells by injecting parental strain thymocytes into irradiated  $F_1$  hybrids and collected the TDL 3-5 days later. Large numbers of activated T cells were found in spleen and Peyer's patches, but very few in the lamina propria. A similar result was obtained by Herbert Monié (pers. comm.), using the same source of activated T cells and labelling with  $^{125}\text{I}$ -UdR, who found 1-4% of activated T cells in the lamina propria compared to 10-20% of normal TDL. These results are in contrast to those obtained by Sprent and Miller (1972b) who also used cells activated by injecting parental thymocytes into irradiated  $F_1$  recipients. The TDL were labelled with  $^3\text{H}$ -thymidine or  $^{51}\text{Cr}$  and they found that after injection into syngeneic recipients the activated cells had a greater affinity for the gut than normal TDL.

There is accordance however that some populations of activated T cells extravasate into areas of inflammation. Lymph node cells removed

4 days after the donor mice had received oxazolone and labelled with  $^{51}\text{Cr}$  or  $^{125}\text{IUdR}$  migrated to inflamed <sup>ears</sup> (Asherson et al., 1973a; Sless & Parrott, pers. comm.). The cells were found to be T lymphoblasts and the propensity for blast cells to migrate rather than the small lymphocytes present in the population was shown by the fact that a greater percentage of  $^{125}\text{IUdR}$  labelled cells migrated to the ears than the same cells labelled with  $^{51}\text{Cr}$ . Blast cells present in the thoracic duct of rats immunised against Listeria, do not recirculate (McGregor et al., 1971) and move to sites of inflammation (Koster et al., 1971; Koster & McGregor, 1971). Rosentreich et al. (1971) showed that peritoneal exudate cells in immunised guinea pigs were short lived cells able to mediate delayed hypersensitivity, providing indirect evidence that T lymphoblasts move to sites of inflammation.

The long term fate and function of these cells is unknown, although some have been shown to transfer adoptive immunity against the antigen that produced them (Koster et al., 1971; Koster & McGregor, 1971), and Sprent and Miller (1972c) found that activated TDL were specifically cytotoxic in vitro and in vivo.

## 2. Lymphocyte recruitment

Antigen has two discernible effects on lymphocyte circulation: one is the non-specific retardation of cell traffic through the stimulated lymph node or spleen, the second is the specific selection of antigen sensitive cells. The relationship between these phenomena is not clear.

## 2a. Specific recruitment

The most convincing experiments which demonstrate selective recruitment <sup>are</sup> ~~and~~ those that show selective depletion of antigen reactive cells in the circulating pool 24-36 hours after antigen administration. TDL collected 24-36 hours after the intravenous injection of sheep erythrocytes were unable to transfer a primary response to irradiated recipients, although the response to a non-cross-reacting antigen was not impaired in mice (Sprent et al., 1971) and rats (Rowley et al., 1972). The response was restored five days later. Similarly, circulating H2 reactive cells were depleted in mice or rats stimulated with histocompatibility antigens. Parental TDL from mice (Sprent et al., 1971) or rats (Rowley et al., 1972) that had received  $F_1$  cells 2 days earlier gave a depressed graft vs host response when injected into the  $F_1$  strain that had been used to immunise but gave a normal response in a third party hybrid recipient. The response was restored when TDL were removed and tested 3 days after the injection of  $F_1$  cells, and gave a heightened graft vs host response when removed 5 days after immunisation. The reciprocal experiment to the above, which demonstrated the same phenomenon, involved draining the TDL from  $F_1$  rats that had received parental cells 1-2 days earlier. 75% of the TDL were of parental strain origin, and gave a reduced graft vs host activity against the  $F_1$  recipient strain, but not a third party  $F_1$  (Ford & Atkins, 1971). The spleen and lymph nodes of the hybrid recipients had therefore filtered and removed specific H2 reactive cells from the circulation. The spleens from irradiated rabbits which had been injected with bone-marrow cells from normal rabbits produced plaque forming cells in response to sheep erythrocytes and horse erythrocytes, however the



ability of bone-marrow to transfer responses to sheep red cells was abrogated by giving the bone-marrow donors sheep red cells 8-24 hours before transfer. This treatment did not depress the response to horse red cells (Abdou & Richter, 1969). This experiment suggests that the bone-marrow of these rabbits contained immunocompetent cells that were recruited 8-24 hours after the intravenous injection of antigen. The difficulty in interpreting these experiments in terms of the recruitment of antigen reactive cells is the possibility that some other factors could be involved. The transience of the phenomena however supports the recruitment explanation rather than some form of tolerance.

A more direct method of demonstrating the retention of cells in the stimulated organ at the expense of the circulating population is to use a double isotopic labelling technique. Atkins and Ford (see Ford, 1975) labelled parental strain lymphocytes with either  $^3\text{H}$ - or  $^{14}\text{C}$ -uridine and injected them into  $F_1$  recipients together with an unresponsive population ( $F_1$  hybrid or tolerant parental lymphocytes) labelled with the alternative isotope. By 24 hours the label of the responsive population was always in surplus in the spleen and deficient in the thoracic duct lymph.

These experiments demonstrate that unprimed T cells are specifically recruited. There is no real evidence that unprimed B cells are recruited or that they circulate (Strober & Dilley, 1973a, 1973b). The question of specificity has also been applied to the recruitment of primed cells, where both T and B cells are involved. (Rowley et al.,

1972). Experiments have demonstrated the specific depletion of antigen primed cells in the circulation following antigenic stimulation. The TDL from a rat that had been primed to tetanus toxoid were unable to transfer a secondary response to an irradiated recipient if the donor had received tetanus toxoid 24 hours before transfer. The spleen, however, transferred the response during this period (Rowley et al., 1972). TDL from donors immunised to tetanus toxoid and swine influenza virus were added to the perfusate of an isolated spleen that had received tetanus toxoid or a control antigen 3 hours previously. Cells specifically responsive to tetanus toxoid accumulated in the spleen over a 10 hour period. This was shown by the inability of the perfusate to transfer a haemolysin response to tetanus toxoid, and the ability of perfused spleen fragments to transfer the response (Ford, 1972). Although this experiment demonstrated by functional means the recruitment of cells responsive to tetanus toxoid, a double labelling experiment failed to confirm the phenomenon. Ford (1972) labelled cells primed to tetanus toxoid with  $^3\text{H}$ -uridine and normal cells with  $^{14}\text{C}$ -uridine and compared their accumulation in the isolated spleen 24 hours after challenge with tetanus toxoid. He found the ratio of radioactivity produced by immune cells to the amount of radioactivity associated with normal cells was consistently unity. Possible explanations for the failure of this experiment to reveal specific recruitment could be that the donors were labelled several weeks after immunisation, and thus many non-immune cells would also become labelled. There may therefore have been too few labelled antigen reactive cells; also  $^3\text{H}$ -uridine could underestimate the contribution made by specific B cells. Other double labelling experiments have

however detected differences in the recruitment of immune and non-immune lymphocyte populations. Emeson and Thursh (1971) compared the distribution of immune and non-immune lymphocytes that had been immunised to H2 antigens and heterologous red cells (Thursh & Emeson, 1972) and labelled in vivo with  $^3\text{H}$ -thymidine or  $^{14}\text{C}$ -thymidine. Each recipient received memory cells to one antigen (for example, sheep red cells) labelled with  $^3\text{H}$ -thymidine and memory cells to the other antigen (chicken red cells) labelled with  $^{14}\text{C}$ -thymidine. The recipients were injected subcutaneously (or grafted in the H2 experiment) with sheep erythrocytes in one flank and chicken red cells in the contralateral flank. They measured the ratio of label associated with the immune population to that associated with the non-immune population in the lymph nodes draining both flanks. The 'non-immune' population was the immune population to the alternative antigen. After 24 hours they found memory cells to one antigen had preferentially accumulated in the lymph nodes that had been stimulated to that antigen. If there had been no preferential accumulation of cells, the ratio of label from immune to non-immune cells would have been unity. They found a ratio in the H2 system of 1.08 or 1.06 and in the erythrocyte system of  $1\frac{1}{2}$  or 1-2. Thus although the figures were significantly different from the Null hypothesis ( $P < 0.01$ ) they showed that specific accumulation accounts for only a small proportion of the cells retarded by the draining lymph nodes.

## 2b. Non-specific recruitment

Antigen-induced retardation of cell traffic through a lymph node was first noticed by Hall and Morris (1962, 1963, 1965) who reported

a depression in the number of cells in the efferent lymph draining sheep popliteal nodes. They found a 50% decrease in cell number within 30 mins of the subcutaneous administration of antigen, which fell to 20% between 2-3 hours, and was restored to normal 5 hours after antigen (Hall & Morris, 1965). Although this effect could be due to a reduced entry of lymphocytes from the blood, Ford (1969) showed that a large dose of sheep red cells did not alter the entry of cells from the perfusate into an isolated spleen, but did inhibit their exit. Subsequent experiments utilised isotopically labelled lymphocytes which were infused into syngeneic recipients which had been immunised. An accumulation of radioactivity in the stimulated lymphoid tissue was subsequently found (Zatz & Lance, 1971a, 1971b; Emeson & Thursh, 1971; Ford, 1972; Thursh & Emeson, 1972; Frost & Lance, 1974a, 1974b).

Antigen that is injected intravenously or intraperitoneally attracts cells to the spleen and antigen that is administered subcutaneously attracts them to the draining lymph nodes (Zatz & Lance, 1971a). The time taken to initiate the 'lymphocyte trap' and the period it lasts varies with the antigen and the experimental design, but antigen-mediated accumulation of lymphocytes can be detected as early as 30 mins after antigen (Hall & Morris, 1965; Zatz & Lance, 1971a) and may still be present after 72 hours (Zatz & Lance, 1971a). Greatest accumulation probably occurs between 12-48 hours after antigen (Zatz & Lance, 1971a; Ford, 1968; Thursh & Emeson, 1972). A variety of antigens elicit the recruitment of circulating cells. Hall & Morris (1965) using sheep, demonstrated that human serum albumin, ovalbumin, Salmonella typhi

organisms, chicken red cells, Listeria monocytogenes, and Ascaris lumbricoides were effective. By measuring the accumulation of <sup>51</sup>Cr labelled lymphocytes in mice, Frost and Lance (1974a, 1974b) found the soluble antigens bovine gamma globulin, bovine serum albumin and tetanus toxoid to be ineffective in unprimed animals, but to cause cell trapping in primed animals. These results appear to be at variance with those of Hall and Morris (1965). Other antigens that cause trapping are allografts (Emeson & Thursh, 1971; Frost & Lance, 1974a), sheep red cells (Thursh & Emeson, 1972; Ford, 1968, 1969; Frost & Lance, 1974a), chicken red cells (Thursh & Emeson, 1972), keyhole limpet haemocyanin, Salmonella H antigen, pneumococcal polysaccharide III, tumour extracts, xenogenic sera, heat agglutinated bovine gamma globulin, and xenogenic grafts (Frost & Lance, 1974a).

Conventional immunogens are not the only substances that cause accumulation of circulating lymphocytes in locally stimulated lymphoid tissue, other substances include adjuvants (Taub et al., 1970; Dresser et al., 1970; Frost & Lance, 1974b) and particles such as latex, silica, carbon and carageenan (Frost & Lance, 1974b). The duration, lasting several weeks, of trapping caused by adjuvants is prolonged compared to that caused by antigens. The magnitude is similar to that produced by strong immunogens with the exception of C. parvum which shows particularly strong trapping of a phasic nature. It has been postulated (Frost & Lance, 1974b) that adjuvants may in part owe their adjuvanticity to their ability to trap cells. This hypothesis must remain valid until the discovery of an adjuvant, as measured by its effect on the antibody response to a weak immunogen,

which does not cause the local accumulation of circulating cells. The magnitude of trapping caused by particles is similar to that elicited by immunogens, and the duration is variable.

Another effect antigen or antigen-cell interactions may produce that could facilitate the exchange of cells between blood and lymph node is to increase the permeability of the local vascular system (Kelly et al., 1972; Hay et al., 1973; Hay, 1973). During a primary response, not only vascular dilation, but redistribution and enlargement of the lymph node vascular network occurs (Herman et al., 1972). Whether these changes occur rapidly enough to account for the increase in cell recruitment within one hour of antigen is questionable.

## 2c. Mechanisms of cell recruitment

To understand the mechanisms of cell recruitment it is important to elucidate the relationship between the non-specific and the specific phenomena. A gross alteration in cell traffic would allow more time for the selective mechanism to select antigen reactive cells. This argument is only valid if the normal rate of movement of cells through a stimulated organ is too slow to allow the efficient depletion that has been demonstrated. It is possible that the retardation of lymphocytes that are traversing the substance of the node or spleen serves some other function, for example, it has been shown that a tightly packed anoxic environment favours blast transformation (Moorhead et al., 1967). At present too little is known to confidently relate the specific and non-specific phenomena.

Non-specific recruitment, whatever its function, is undoubtedly mediated by antigen. The possible types of interaction are as follows:

- (i) Interaction between lymphocytes and antigen, within or outside the lymph node, that releases some factor which alters lymph node architecture and hinders the movement of cells.
- (ii) Interaction between antigen and macrophages, resulting in an alteration of macrophage structure, for example swelling that could block the sinus and impede cell movement.

Some experiments suggest that lymphocytes are not of vital importance in initiating cell trapping. The magnitude of trapping found in mice which were treated with 1500R or high doses of corticosteroids or antilymphocytic sera and mice which were irradiated and bone-marrow reconstituted and mice which were irradiated, bone-marrow reconstituted and again irradiated, was found to be unaltered by these treatments (Frost & Lance, 1974a). This type of experiment, however, only shows that intact lymphocytes within the lymph nodes are not essential, it does not exclude the possibility that it may require very few cells to interact with antigen and release a trapping substance. Thus labelled cells could initiate their own trapping, with exponential kinetics. It has been suggested that soluble factors produced during lymphocyte activation, such as migration inhibition factor (MIF), may regulate lymphocyte traffic in the draining lymph node. MIF has been detected in sheep, in the efferent lymph from popliteal nodes which were draining a tuberculin (Hay et al., 1973) and a Concanavalin A injection (Hay, 1973); and in the rabbit, in the afferent lymph which

was draining a diphtheria toxoid injection (Kelly et al., 1972).

Kelly et al. (1972) found that when MIF from guinea pigs was injected into the afferent lymphatics of rabbits, it produced paracortical distension and dense aggregations of cellular plugs in the paracortical sinuses, similar to those described by de Sousa and Parrott (1969). Recently, Frost and Lance (1974b) reported that the subcutaneous injection of MIF into mice caused trapping of labelled lymphocytes. It has been suggested that the soluble factors may act by increasing the number of cells that enter the lymph node through the post-capillary venules. (Kelly et al., 1972; Hay, 1973).

Macrophages may play a role in the recruitment of circulating cells in view of the correlation between the ability of substances to trap cells and their attractiveness to macrophages. Thus soluble proteins which are relatively inefficient at inducing trapping are poorly taken up by macrophages, but particulate matter and high molecular weight substances which are good at inducing trapping are also attractive to macrophages (Unanue & Cerottini, 1970). Although a simple explanation for the non-specific recruitment of circulating cells implies a physical blocking of the lymph node sinuses, it is interesting that the flow of efferent lymph is at no time restricted (Frost & Lance, 1974b; Hall, 1974). This observation may indicate a more subtle method of impeding the lymphocytes' progress.

Theories on how lymphocytes are selectively recruited are compounded by ignorance of where they are selected. Although a special affinity between lymphocytes and the endothelial cells of the post-capillary



venules has been suggested (Gowans & Knight, 1964; Gesner, 1966), there is no real evidence that they regulate the entry of lymphocytes. The post-capillary venules are obviously major entry points, because their endothelial cells which are normally high cuboidal become very thin in the lymphocyte depleted cortex of neonatally thymectomised mice (Parrott et al., 1966) or in the homozygous nu/nu mouse (de Sousa et al., 1969). The thin flattened walls rapidly assume a normal appearance after the intravenous injection of lymphocytes (Goldschneider & McGregor, 1968b). Although Hall and Morris (1962, 1963) found few cells in the afferent lymph of sheep popliteal nodes, Kelly (1970) found many cells in the afferent lymph of rabbits draining an intradermal depot of antigen, and demonstrated their localisation in the popliteal node. Thus while the post-capillary venules may be the main point of entry of recirculating cells, afferent lymphatics afford an entry route for cells draining the dermis. An interesting experiment by McConnel et al. (1974) showed that cannulation of the efferent lymphatic from a single popliteal node of a sheep primed to PPD over a 4 week period, combined with repeated injections of PPD, resulted in the specific abrogation of the systemic response to PPD. Thus efferent cannulation caused the disappearance of antigen specific cells <sup>but non-specific cells</sup> had escaped into the circulation. These results suggest either that antigen specific cells are recruited from the post-capillary venules, allowing non-specific cells to pass on, or that cells are non-specifically recruited into the substance of the node, where specific cells are retained and non-specific cells are returned to the blood. This implies that some cells exit via the post-capillary venules. Additional evidence that a selective screening mechanism exists at the level of the endothelial

cells lining the post-capillary venules was derived from experiments in sheep which showed the specific depletion of MLC reactive cells in the efferent lymph of both stimulated and contralateral nodes within 30 hours of administration of allogeneic cells (Hay et al., 1974). These authors point out that an alternative explanation (which might also apply to the McConnel experiment) is the production of suppressive factors; thus the specific cells would be present in the circulation, but unresponsive.

Any selective screening function performed by endothelial cells implies the presence of tightly bound membrane antigen. At present, information about the physiology of the endothelial cell membrane is lacking due to the difficulties involved in working with this tissue. The endothelial cells could also play a role in the non-selective recruitment of cells, if their permeability could be altered by various factors. One of the problems about crediting the endothelial cells of the post-capillary venules with a special responsibility for regulating lymphocyte traffic is that another mechanism has to be invoked for the spleen, where post-capillary venules are absent.

Various experiments have shown that modifying the membrane of lymphocytes with various enzymes changes their migration pattern. Concanavalin A (Gillette et al., 1973) caused the redistribution of lymphocytes from the lymph nodes to the spleen, and trypsin-treated cells (Woodruff & Gesner, 1968) showed deficient localisation in lymph nodes. Various experiments have demonstrated that the removal of sialic acid residues by glycosidases (Gesner & Ginsburg; Gesner, 1966), neuraminidase

(Woodruff & Gesner, 1969) or Newcastle Disease Virus (Woodruff & Woodruff, 1972a, 1974) produced a decrease in the number of lymphocytes that migrated to the lymph node and spleen and produced an increase in the number of cells found in the liver. Glycoproteins are however ubiquitous structures on the surfaces of mammalian cells and they almost certainly play an important role in many processes that involve interactions between cell membranes (Cook, 1968). The possibility therefore that the failure of these treated lymphocytes to enter lymph nodes is a secondary effect of the removal of glycoproteins, due for example to increased susceptibility of the cells to sequestration by the liver, cannot be excluded.

### 3. Labelling techniques for studying cell localisation

A variety of radio-isotopes are at present available to label lymphocytes and allow their localisation in vivo to be followed. Various cell constituents may be labelled - DNA, RNA and protein. An ideal isotope for labelling cells would have the following properties:

- (i) All lymphocytes would be labelled with equal intensity; the isotope would not favour one cell population above another. Alternatively, the isotope would be highly and reproducibly selective for an identifiable sub-population.
- (ii) The incorporation of the isotope would be stable, and it would not elute from lymphocytes during the period of study.
- (iii) Any isotope that is released from dead or damaged cells would be in a form that is not reutilisable by other cells.
- (iv) The process of labelling lymphocytes would not affect the immunocompetence or behaviour of the lymphocytes.

No isotope studied to date possesses all of these properties. The important point is to realise how far these characteristics apply to the particular isotope in question, and to match the isotope with the requirements of the experiment. Physical properties of the isotope such as the half-life and whether it decays by  $\beta$  or  $\delta$  emission are other details to be considered. The advantages and limitations of some isotopes which are commonly used to label lymphocytes are described below.

### 3a. $\beta$ Emitters

#### (1) Isotopes that label DNA

$^3\text{H}$ -thymidine and  $^{14}\text{C}$ -thymidine are incorporated into proliferating cells during the DNA synthetic phase of their cycle. They can be used to label cells in vitro or in vivo. When used in vitro only large lymphocytes will become labelled, although the percentage which are labelled depends on the time of incubation. Labelling in vitro has been used to study the localisation of large lymphocytes (Hall et al., 1972). When used in vivo, different regimes of  $^3\text{H}$ -thymidine administration can distinguish between long-lived and short-lived cells (Everett & Tyler-Caffrey, 1967). A recommended regime for labelling long lived and short lived lymphocytes in the rat is to give daily injections of  $^3\text{H}$ -thymidine for 20 days or 5 days and allow some time interval before harvesting the cells (Ford & Hunt, 1973). Some newly formed long-lived lymphocytes will be present after 5 days of labelling. Using this technique, the localisation pattern of long-lived and short-lived lymphocytes has been studied (Parrott & de Sousa, 1971) and the migration of short-lived cells to the peritoneal exudate of rats was

observed (Koster & McGregor, 1971). The disadvantages of the  $^3\text{H}$ -thymidine label is that some label released from dead cells is re-utilised (Feinendegen et al., 1966; Mitchell et al., 1963; Robinson et al., 1965) and in high enough concentrations the  $^3\text{H}$ -thymidine can damage cells and inhibit their proliferation. (McGregor, 1969).

(ii) Isotopes that label DNA and RNA

$^3\text{H}$ - and  $^{14}\text{C}$ -labelled adenosine, guanosine and cytidine label RNA and DNA and uridine which labels RNA may also become incorporated into DNA (Comings, 1966). The most commonly used of these isotopes are  $^3\text{H}$ -adenosine (Gowans, 1962; Gowans & Knight, 1964; Parrott & de Sousa, 1966) and  $^3\text{H}$ -uridine (Ford, 1969; Ford & Simmonds, 1972; Howard et al., 1972; Gutman & Weissman, 1973; Sprent, 1973).  $^{14}\text{C}$ -uridine has been used (Ford & Simmonds, 1973) as has  $^3\text{H}$ -cytidine (Sainte-Marie and Peng, 1974).  $^3\text{H}$ -guanosine is rarely used, possibly because it is somewhat insoluble (W.L. Ford, pers. comm.). Gowans (1962) using  $^3\text{H}$ -adenosine noticed an uneven distribution of label amongst small lymphocytes, and this phenomenon was studied by Howard (1972) who demonstrated that  $^3\text{H}$ -uridine was incorporated into T cells from rats about 15 times as heavily as into B cells. This preference for T cells has been found to be characteristic for all the nucleosides that are incorporated into RNA. (Howard, unpublished results). Thus, when using a  $^3\text{H}$ -nucleoside to label a heterogeneous lymphocyte population in vitro, large lymphocytes and T cells will be more heavily labelled than B cells.  $^3\text{H}$ -uridine appears to have some marginal advantages over the other nucleosides in terms of the quantity that is incorporated (W.L. Ford, pers. comm.). Approximately half the radioactivity is lost from cells

within twelve hours of labelling, and is subsequently lost more slowly at about 20% a day due to the metabolic turnover of RNA (Ford & Hunt, 1973). 50% of the activity was recovered in cell-free thoracic duct lymph or urine of rats which had been injected 24 hours previously with  $^3\text{H}$ -uridine labelled thoracic duct cells (Goldschneider & McGregor, 1968b). Similarly, G. Rannie (pers. comm.) recovered 23% of the injected dose in the perfusate of a rat which had been injected 24 hours previously with  $^3\text{H}$ -uridine labelled thoracic duct cells.

#### (iii) Isotopes that label proteins

$^3\text{H}$ -leucine was used by Gowans (1962) for autoradiographic estimations and Black (1974) for quantitative localisation studies. It labels more than 95% of all the small and large lymphocytes in autoradiographs exposed for 14 days. Information about its rate of elution and reutilisation is lacking, but the suggestion that it does elute in vivo stems from the fact that 32.1% of the injected dose was recovered from the perfusate of a rat that had been injected with labelled thoracic duct cells 24 hours previously (G. Rannie, pers. comm.).

### 3b. Emitters

#### (1) Isotopes that label DNA

$^{125}\text{I}$ UdR is incorporated into DNA by dividing cells (Hughes et al., 1964). It has been used to label dividing cells in vitro (Hall et al., 1972; Asherson et al., 1973a) and in vivo (Asherson et al., 1973a). It does appear to be more stable than the isotopes that are incorporated into RNA or proteins, its rate of elution from tumour cells is impressively low at 4% over 24 hours (P. Chisholm, pers. comm.). Although it has

been claimed that it is not reutilised (Hughes et al., 1964), this is controversial (G. Harris, pers. comm.).  $^{125}\text{I}$ -UdR is toxic in high concentrations,

although H. Monie (pers. comm.) used different concentrations of  $^{125}\text{I}$ -UdR to label rat lymphoblasts and found no difference in their localisation patterns. The disadvantage of  $^{125}\text{I}$ -UdR is that at present it is only used to label blast cells. Any long term regime designed to label long lived lymphocytes might encounter toxicity problems.

(ii) Isotopes that label protein

(ii.a)  $^{51}\text{Cr}$

$^{51}\text{Cr}$  has been used extensively to estimate cell localisation quantitatively (Zatz & Lance, 1970, 1971a; Zatz et al., 1971; Lance & Taub, 1969; Zatz et al., 1973a, 1973b; Dresser et al., 1970; Frost & Lance, 1974a; Zatz & Gershon, 1974; Sprent & Miller, 1972b; Sprent, 1973; Asherson & Allwood, 1972; Asherson et al., 1973a). It has a half-life of 28 days and is bound mainly to intracellular proteins (Rona, 1969). Labelling is, however, uneven, large lymphocytes take up most label (Howard et al., 1972). The elution of  $^{51}\text{Cr}$  from lymphocytes in vitro is high, 50-60% over 24 hours (Rona, 1969). The fate of the eluate in vivo is uncertain. A major proportion will be rapidly excreted (Bainbridge et al., 1966) and although it has been claimed that only 2% of the label released from dead cells can be reutilised by lymphocytes (Bunting et al., 1963; Bainbridge et al., 1966), the possibility that released label may reattach to erythrocytes cannot be excluded. Thus experiments have demonstrated that the red blood cells of rats contained 6% of the injected dose, 24 hours after the intravenous

injection of  $^{51}\text{Cr}$  labelled thoracic duct cells (K. Donald, unpublished results). High concentrations of  $^{51}\text{Cr}$  have been shown to produce an increase in the number of cells that localise to the liver (K. Donald unpublished results).

(11.b) Technetium-99m

Technetium-99m ( $^{99\text{m}}\text{Tc}$ ) has been used to label human red cells (Smith, 1974; Atkins et al., 1972) and various murine and human nucleated cells (Gillespie et al., 1973). When used in conjunction with stannous chloride, it appears to be firmly fixed within the cell, three cycles of freeze-thawing released only 10% of the isotope. Murine sarcoma cells, when incubated for 48 hours failed to release any isotope (Barth & Gillespie, 1974). Cells that have been labelled with  $^{99\text{m}}\text{Tc}$  were found to contain the isotope in the nuclear, mitochondrial and microsomal fractions. Few experiments have been done to date regarding the behaviour of labelled lymphocytes, although it has been shown that labelled cells can incorporate  $^3\text{H}$ -thymidine,  $^3\text{H}$ -leucine and  $^3\text{H}$ -glycine (Barth & Gillespie, 1974). If it were satisfactorily demonstrated that labelling with  $^{99\text{m}}\text{Tc}$  does not affect the immunocompetence of lymphocytes, and when the distribution of label among different lymphocyte populations is established, this would provide a useful label for localisation studies. The half life of six hours which is useful for clinical studies may be somewhat prohibitive for large scale animal experiments.

(11.c)  $^{75}\text{Se}$ -selenomethionine

$^{75}\text{Se}$ -selenomethionine is an analogue of methionine in which sulphur is replaced by selenium. The isotope of selenium,  $^{75}\text{Se}$ , has a half-life



of 120 days. Studies to be reported in Chapter IV reveal that the amino-acid is incorporated in vitro by a variety of murine lymphocytes into the TCA-precipitable fraction of cells. The elution, reutilisation and effect of the isotope on lymphocyte immunocompetence are investigated in Chapter IV and the results suggest that  $^{75}\text{Se}$ -selenomethionine may provide a useful isotope for lymphocyte localisation studies.

The experiments which are reported in Chapter V investigate the kinetics of lymphocyte localisation in response to oxazolone. Two features of these experiments make the use of a  $\gamma$  emitter desirable:

- 1) Differences in the amount of localisation are to be estimated.
- 2) The experiment requires treatment of lymph nodes from 120 mice.

The advantages of a  $\gamma$  emitter over a  $\beta$  emitter are the ease of tissue preparation and the consequent reduction in experimental error. These advantages are especially beneficial when large numbers of lymph nodes are to be treated.

The experiments which are performed in Chapter V require that a non-dividing cell population is labelled, thus excluding the use of  $^{125}\text{I}$ -UdR.  $^{51}\text{Cr}$  was not used because of the uncertainty of its effect on lymphocyte immunocompetence and the possibility that it labels red cells. It was decided to investigate the use of a  $\gamma$  emitting amino-acid,  $^{75}\text{Se}$ -selenomethionine as a possible alternative  $\gamma$  emitting isotope.

## CHAPTERS IV & V

### MATERIALS AND METHODS

#### 1. Mice

Three to four month old male mice of the inbred strain CBA/H, C57Bl/10 and (CBA/H x C57Bl/10) F<sub>1</sub> were used. Thymus donors were six to ten weeks old. 6 week old nu/nu mice were kindly provided by Dr. Helen Pritchard.

#### 2. X-irradiation

X-irradiation was administered with a Westinghouse machine under the following conditions; 230kV; 15 MA; 0.5mm Cu and 10mm Al filtration; dose rate 66r/min.

#### 3. Cell suspensions

Lymph nodes and thymuses were gently homogenised in ground glass tubes, and filtered through steel mesh. Bone marrow cells were extruded from femurs and humeri using a 1ml syringe and a 27g needle. The source of lymph node cells were the axillary, brachial, inguinal and mesenteric lymph nodes. All suspensions were prepared in Hanks BSS (pH 7.4, adjusted with 1.4% sodium bicarbonate), at 0-4°C. Viability was estimated with nigrosin and the cell volume was adjusted to the required number of cells per recipient in 0.4mls, and injected into the tail vein.

#### 4. Oxazolone

2-ethoxy-methylene-4-phenyl oxazolone (B.D.H.) was dissolved in absolute alcohol at 60°C and 10% w/v. In Chapter IV, 5mg of oxazolone was applied to a shaved area of skin on the left flank in order to stimulate mainly the axillary and brachial nodes. In Chapter V, mice were anaesthetised with Nembutal and 5mg of oxazolone was applied to the left hind footpad, which was subsequently covered with a plaster of Paris bandage ("Gypsona", T.J. Smith & Nephew Ltd., England) to localise the response mainly to the popliteal and inguinal nodes. Controls received 0.1mls of absolute alcohol on the shaved flank in Chapter IV, and to the left footpad of anaesthetised mice in Chapter V.

#### 5. Measurement of DNA synthesis

DNA synthesis was measured by uptake of <sup>125</sup>I-labelled 5-iodo-2'-deoxyuridine (<sup>125</sup>IUdR) (Radiochemical Centre, Amersham) after the method of Hughes *et al.* (1964) modified by Pritchard and Micklem (1972). Mice received  $5 \times 10^{-8}$  moles of 5-fluorodeoxyuridine (FUDR) (Roche products, Ltd.) in 0.2 mls saline ip followed one hour later by 1μCi of <sup>125</sup>I-UdR, adjusted to a specific activity of 5mCi/mg, by the same route. Mice were killed two hours later and their nodes fixed in formal-ethanol (4% formalin and 70% ethanol). Unincorporated label was removed by several changes of 70% ethanol over a period of 5-7 days, until the washings contained no more than twice background counts (Elkins, 1970). In Chapter IV the axillary and brachial nodes from one side were counted together, and in Chapter V the inguinal and popliteal nodes were counted individually for 5 mins in a Packard Gamma counter. The injected dose was counted for one minute. The

background counts were subtracted from the organ counts, and the counts per minute were adjusted to the counts that would have been obtained from an injected dose of  $10^6$  cpm. The calculation was therefore as follows:

$$^{125}\text{I-UdR uptake} = \log_{10} \left[ \frac{\text{cpm from organ} \times 10^6}{\text{injected dose in cpm}} \right]$$

It can be seen that the terms cpm cancel out, and the  $^{125}\text{I-UdR uptake}$  is the  $\log_{10}$  of the proportion of the injected dose that was incorporated.

#### 6. Plaquing technique

The 19S and 7S response of transferred spleen cells in irradiated recipients to sheep erythrocytes (Sheep cells in Alsevers', Tissue Culture Services Ltd., Slough) was tested by a modification (Wortis et al., 1966; Dresser & Wortis, 1967) of the Jerne plaque technique (Jerne et al., 1963). Agarose Indubiose, A37, FF9760 (L'Industrie Biologique Francaise, Gennevillies, France) was used for top and bottom layers. The cells were prepared in phosphate buffered saline (Mishell & Dutton, 1967).

#### 7. Labelling techniques

##### (a) $^{75}\text{Se-L-selenomethionine}$

##### (i) The isotope

$^{75}\text{Se-L-selenomethionine}$  ( $^{75}\text{Sem}$ ) was obtained from the Commissariat a L'energie Atomique (91-Gif-Sur-Yvette, France; British importers are Micro-Bio Ltd., London). The specific activity varied from 21mCi/mg -

68mCi/mg. The half life of  $^{75}\text{Se}$ -selenium is 120 days. The isotope was supplied at a concentration of 500 $\mu\text{Ci/ml}$  and was diluted for use to 100 $\mu\text{Ci/ml}$ .

(ii) The medium

The medium which was used for labelling mouse lymphocytes was Hanks BSS, pH 7.4, containing 5% foetal calf serum (Biocult Labs., Ltd.) and 2% Minimal Essential Medium vitamins (Biocult Labs. Ltd.) and 200 units/ml of a streptomycin/penicillin mixture (Biocult Labs. Ltd.). The medium which was used to label rat thoracic duct cells was phosphate buffered saline (Mishell & Dutton, 1967) enriched as above.

The concentration of isotope which was routinely used was 2 $\mu\text{Ci}/10^7$  cells. Lymphocytes were washed three times in Hanks BSS, and added to the medium containing isotope to give a final concentration of  $2 \times 10^7$  cells/ml. The pH of the medium sometimes became more acid on addition of the isotope (depending on specific activity). This was corrected by the addition of 4.5% sodium bicarbonate (Biocult Labs. Ltd.). The cultures were gassed with a 10%  $\text{CO}_2$ /Air mixture before being incubated for 60 mins at  $37^\circ\text{C}$  in a gently shaking water bath. At the end of incubation, cells were washed three times in Hanks BSS at  $0-4^\circ\text{C}$ . The supernatant from the last wash was usually retained. After washing, viability of labelled cells was assessed using the nigrosin dye exclusion test. Cell suspensions were adjusted to a concentration of usually  $12.5 \times 10^6$  cells/ml, and 0.4ml aliquots injected into the tail vein. An injection dose was retained to count the radioactivity.

### (iii) Removal of organs

At various times after injection of labelled cells mice were killed and a variety of lymphoid tissue and non-lymphoid tissue was removed. The lymph nodes which were excised were the pairs of axillary, brachial and inguinal nodes and the mesenteric nodes. The paired axillary and brachial nodes were counted together, the paired inguinals were counted together and the three mesenteric nodes were counted together. Other organs that were excised were the spleen, liver, both femurs, both lungs, the whole thymus and a known volume of blood removed from the retroorbital plexus. In some experiments the blood was separated by centrifugation and the plasma counted separately from the cells. When the intestine was counted it was cut into manageable sections. Peyer's patches are included in the intestinal counts. The organs were counted immediately after extirpation, with no tissue preparation, in the Packard gamma counter.

### (iv) Expression of results

Experiments utilised groups of 4 to 6 mice. The mean cpm for each organ per group was calculated and expressed as a percentage of the injected dose. The standard deviation was also expressed as the percentage of the injected dose. When the results are expressed as the 'total lymph nodes', this figure was arrived at by taking the counts in the paired axillary, brachial, inguinal and 3 mesenteric nodes, and multiplying by 2 (Zatz & Lance, 1970). In some experiments where quantitative differences were to be estimated, only the mean of the cpm was recorded. In the experiments using rats, results were expressed either as the percentage of the injected dose per gram of tissue, per

ml of fluid,) or as the percentage of the activity found in the lymph nodes.

(b)  $^3\text{H}$ -methionine

$^3\text{H}$ -L-methionine (Radiochemical Centre, Amersham, Bucks.), specific activity 55mCi/mg, was incubated with mouse lymphocytes at a concentration of 2 $\mu\text{Ci}/10^7$  cells. The labelling procedure was as described for  $^{75}\text{Se}$ .

(c) L-4, 5- $^3\text{H}$ -leucine

$^3\text{H}$ -leucine (Radiochemical Centre, Amersham, Bucks), specific activity 420mCi/mg was incubated with mouse lymphocytes at a concentration of 10 $\mu\text{Ci}/10^7$  cells. The labelling medium and subsequent washings were as described for  $^{75}\text{Se}$ .

8. Experiments utilising rat thoracic duct cells

These experiments were performed in collaboration with Ms. P. Chisholm and Dr. G. Rannie, in the laboratory of Dr. W.L. Ford (Pathology Dept., Edinburgh). The thoracic duct of male adult rats of the inbred strain A0 were cannulated and circulating lymphocytes were collected by the technique described by Ford and Hunt (1973).

(a) 24 hour cultures of thoracic duct cells

Thoracic duct cells were labelled with  $^{75}\text{Se}$ , washed 3 times and resuspended at various concentrations in RPMI 1640 medium (Biocult Labs. Ltd.) supplemented with 20mM Hepes, 0.8gm/l sodium bicarbonate, glutamine, non-essential amino acids, sodium pyruvate, penicillin/

streptomycin and 10% foetal calf serum. They were cultured for 24 hrs in 1 ml aliquots in the wells of Linbro tissue culture plates (SB-16-24-TC multi-dish disposal trays) which were covered and incubated at 37°C on a gently rocking platform.

(b) 24 hr cultures of rat tumour cells

WF/G1 tumour cells, an in vitro established cell line from a Gross virus-induced lymphoma of Wistar-furth rats, were labelled with <sup>75</sup>Se, and cultured at  $5 \times 10^4$  cells/ml in 1 ml aliquots under the same conditions described above for the thoracic duct cells.

(c) Perfusion of rats

Rats that had been injected with labelled thoracic duct cells were perfused 24 hrs after the injection of cells prior to the removal of organs. The chest and abdominal cavities were opened and the animals were perfused with phosphate buffered saline for 30 mins. through a cannula passing into the left auricle and with the vena cava severed to allow collection of the perfusate from the abdominal cavity.

9. Autoradiography of lymphocytes labelled with <sup>3</sup>H-methionine

Lymphocytes labelled with <sup>3</sup>H-methionine were adjusted to a concentration of  $2.5 \times 10^6$  cells/ml, and 0.2ml aliquots were gently spun on to clean slides in a cytocentrifuge (Shandon Co. Ltd.). The slides were dried in air and fixed overnight in methanol. They were then dried and dipped in liquid gel emulsion (Ilford, K2 Nuclear Research Emulsion in Gel form), again dried in air and stored in black sealed boxes at 0-4°C. A smear of non-radioactive sample was treated similarly to serve as a background



control. The autoradiographs were left for 15-17 days before developing (Kodak, D19) and fixing. They were immediately placed in 10% formaldehyde for 10 mins to prevent wrinkling of the autoradiographic film, rinsed in tap water for 30 mins, and dried in air overnight. Leishman's stain was used to stain the slides.

#### 10. Estimation of $^3\text{H}$ -leucine incorporation

In Chapter V, lymph node lymphocytes were labelled with  $^3\text{H}$ -leucine and injected into syngeneic recipients. The proportion of lymphocytes localising in the lymph nodes was estimated by homogenising the nodes in cold 10% trichloroacetic acid (TCA) to precipitate the protein. The precipitate was filtered and collected on Whatman glass fibre filter paper, and allowed to dry at  $37^{\circ}\text{C}$  overnight. 3 to 4 mls of 0.5% Butyl PBD scintillator in toluene was added to each sample and the amount of radioactivity present was measured in a Packard liquid scintillation counter.

## CHAPTER IV

### USE OF $^{75}\text{SE}$ -SELENOMETHIONINE AS AN ISOTOPIC MARKER FOR CELL LOCALISATION STUDIES. EVALUATION OF THE TECHNIQUE

#### RESULTS

In this chapter experiments were performed to investigate the parameters of incorporation of  $^{75}\text{Sem}$  by various cell types, and the properties of the labelled cells with a view to evaluating the potential of the isotope as a cell marker in cell localisation studies.

#### A. Parameters of incorporation of the isotope

##### 1. Kinetics of incorporation. Fig. IV.1.

Mouse lymph node lymphocytes were incubated in 10 ml volumes with  $^{75}\text{Sem}$  at concentrations of  $1\mu\text{Ci}$ ,  $5\mu\text{Ci}$  and  $10\mu\text{Ci}/10^7$  cells. Aliquots were removed after incubation for 60 mins, and 10 mls of isotope-free Medium 199 (Biocult Ltd.) was added immediately after the removal of the second aliquot (arrow on Fig. IV.1). Another aliquot was removed immediately, and after the subsequent time intervals shown. The aliquots were estimated for cell viability, and the supernatant removed. The pellet was washed three times and counted for radioactivity. Although each sample was expected to contain about  $10^7$  cells, only about  $2 \times 10^6$  cells were present. The uptake did not increase proportionately with the concentration of isotope. After 60 mins of incubation approximately 11,000 cpm were present in  $2 \times 10^6$  cells incubated with  $1\mu\text{Ci}$ , 18,000 cpm

with 5 $\mu$ Ci and 40,000 cpm with 10 $\mu$ Ci. There was no detectable decrease in the counts present in the cell pellets over a ten hour period. This does not mean that there was no elution of label over this time period. It was discovered in retrospect that the cold medium which was added after 1 hr only contained enough methionine to dilute the pool of isotopic selenomethionine by 1/2. Thus a continual process of loss and uptake of  $^{75}\text{Se}$ m, if it were occurring, would not be inhibited to a noticeable extent, by the presence of a relatively small quantity of cold methionine.

2. Incorporation into the TCA insoluble cellular fraction. Fig. IV.2

Mouse lymph node cells were incubated in 10 ml volumes with 2 $\mu$ Ci/ $10^7$  cells, and an aliquot containing  $10^7$  cells was removed after 60 mins. Isotope-free Medium 199 was added after the first aliquot had been removed. Further aliquots were removed at the times shown in Fig. IV.2. The cell viability of the aliquots was estimated. The cells were washed 3 times, the supernatants discarded, and the final cell pellets counted for radioactivity. The same pellets were then homogenised in 2mls of cold TCA. The precipitates which formed were deposited on glass fibre filters which were counted for radioactivity. The counts found in the TCA precipitable fractions, that is protein, RNA and DNA, were found to be approximately 75% of the total counts over 24 hrs. The aliquots removed up to 8 hrs contained  $10^7$  viable cells, the cpm decreased at 24 hrs to a value approximately 30% of the initial value, and at 24 hrs the viable cell count had decreased to approximately  $2 \times 10^6$  cells/ml.

### 3. Elution of the isotope. Fig. IV.3

In order to investigate the non-specific elution of  $^{75}\text{Se}$  from labelled viable lymphocytes, a highly viable population of lymphocytes, removed in a way least likely to damage them, was used. Thoracic duct cells from AO strain rats were labelled with  $^{75}\text{Se}$  at a concentration of  $2\mu\text{Ci}/10^7$  cells. After labelling and washing, the cells, which had retained 100% viability, were resuspended in enriched RPMI 1640 medium and cultured at concentrations of  $10^5$ ,  $10^7$  and  $5 \times 10^7$  cells/ml in 1ml quantities. At 1, 2, 6 and 24 hrs after establishing the cultures, the amount of label lost from the cells at their different concentrations was estimated. A 0.5ml sample of supernatant was carefully removed and counted for radioactivity, the remaining cells and supernatant were counted for viability and radioactivity. The percentage of the total counts found in the supernatant was calculated as follows:

$$\% \text{ in supernatant} = \left[ \frac{\text{cpm in total supernatant (cpm in 0.5 ml} \times 2)}{\text{total cpm (cpm in cells} + \text{cpm in 1 ml supernatant)}} \right] \times 100$$

Each point in Fig. IV.3 is the mean cpm from duplicate cultures. It should be noted that as much as 3-9% of the total counts were found in the medium immediately after labelling (time 0). At 2 hrs, 16-19% of the total counts were found in the supernatant, 28-34% at 6 hrs which increased to 54-64% by 24 hrs. It is therefore apparent that over the 24 hr period the cells lost 50-55% of their label. The viability during this period was 95% at 6 hrs, and 85% at 24 hrs. There was no significant difference in the rate of elution from cells incubated at different concentrations, and no difference in their viability.

4. Incorporation and elution of isotope by tumour cells. Table IV.1

W.F. tumour cells were labelled at a concentration of  $2\mu\text{Ci}/10^7$  cells and cultured in the presence and absence of  $10^7$  syngeneic rat lymph node cells. The release of isotope into the supernatant was estimated as described for thoracic duct cells. The results are shown in Table IV.1 where the individual results from duplicate cultures are recorded. They were very variable which was probably due to the fact that only  $5 \times 10^4$  cells were present in each culture representing about 770 cpm. Therefore a release of 10% of the label into the supernatant would have represented only twice background level. From these results it is difficult to conclude how much label was released prior to 24 hrs, but the cells probably released 30-50% of their label over a 24 hr period. The presence of  $10^7$  lymphocytes did not appear to affect the release of label.

5. Incorporation of isotope by different lymphocytes. Table IV.2

Lymph node lymphocytes, thymocytes, cortisone resistant thymocytes, spleen cells and bone-marrow cells were labelled with  $2\mu\text{Ci}/10^7$  cells in duplicate cultures. The spleen cell suspension had been treated with Tris-ammonium chloride to lyse red blood cells. After labelling and washing the cells 3 times, the viability was assessed, supernatant removed and the cells counted for their radioactive content. The results are shown among the results of Table IV.2 and are coded Se VI. The bone-marrow cells and thymocytes produced higher counts than other lymphocytes. Table IV.2 presents a summary of the results of labelling lymphocytes, in terms of isotope incorporation, derived from all the experiments which were performed using  $^{75}\text{Se}$ . The specific activity

and the batch number of isotope is included to detect if there is any correlation between specific activity and the amount of isotope incorporated. In the third experiment (Se 111) gassing with a  $\text{CO}_2$ /air mixture was omitted and the counts associated with these cells were relatively low. A reasonable approximation of the number of counts to be expected from  $10^7$  lymphocytes is 100,000-240,000 cpm. The supernatant from the last wash was found usually to contain about 5% of total counts and never more than 10%.  $2\mu\text{Ci}$  produce about  $4 \times 10^6$  cpm, thus  $10^7$  lymphocytes incorporate about 2-5% of the activity which is provided.

#### 6. Autoradiography of cells labelled with $^3\text{H}$ -methionine

This experiment was designed to ascertain whether  $^3\text{H}$ -methionine labelled all lymphocytes, whether the intensity of labelling varied with lymphocyte populations, and whether there was any correlation between intensity of labelling and size of the lymphocyte. In order to ascertain if both B and T cell populations incorporated  $^3\text{H}$ -methionine, lymph node lymphocytes from CBA mice (T and B cells), lymph node lymphocytes from nu/nu mice (almost all B cells) and thymocytes (almost all T cells) were labelled with  $^3\text{H}$ -methionine and autoradiographs were prepared. The number of cells ~~that~~ containing grains, and the number of grains they contained is shown in Table IV.3. Cells containing less than 5 grains were considered to be unlabelled, by this criterion 97% of CBA lymph node lymphocytes, 91.5% of nu/nu lymph node cells and 97.6% of thymocytes were labelled. The majority of lymph node cells from CBA and nu/nu mice possessed 5-20 grains per cell in contrast to preparations of CBA thymocytes in which 46% of the cells possessed 5-20 grains and 50% contained 20-50 grains. It is possible that the tendency of

thymocytes to show a greater labelling intensity was due to some property of T cells, however it was noticed that the greater number of grain counts were associated with larger cells. Therefore the approximate size of the lymphocyte (small 5-8 $\mu$ , medium 8-10 $\mu$ , large 10 $\mu$ +) was noted with the grain count. The results which are presented in Figs. IV.4a, 4b and 4c show a direct correlation between grain count and cell size. The grains were not observed to be associated with any particular region of the cells (Plates IV.1 to IV.3).

#### B. Reutilisation studies

The experiments in this section were designed to discover whether isotope released from dead and dying cells, or cells killed in situ, for example as a consequence of an immune response, could be reutilised by lymphoid cells.

##### 1. Localisation of supernatant counts. Table IV.4.

The supernatant from the third wash of labelled cells may contain as many as 10% of the total (cells plus supernatant) counts. It is important to know where these counts are found after intravenous injection of labelled cells.  $5 \times 10^6$  labelled lymph<sup>node</sup>/cells containing 35,250 cpm were injected in 0.4ml aliquots into 8 recipients. The supernatants from the third wash contained 3,470 cpm/0.4ml and was injected in 0.3ml quantities into a further 8 recipients. The distribution of radioactivity in various organs was measured at 2 and 24 hrs. At 2 hr 3.2% of the supernatant counts were found in lymph nodes and 2.9% in the spleen. These figures decreased to 2.9% and 1.3% respectively by 24 hrs. The majority of recovered supernatant counts

were found in the liver at both times. Only about 18% of the total activity injected in the supernatant was detected at 24 hrs. Regarding the localisation of the lymphocytes it is interesting to note the redistribution of cells between the spleen and lymph nodes which occurred between 2 and 24 hrs.

## 2. Localisation of cells killed by freeze-thawing. Table IV.5.

The experiment was designed to detect what proportion of dead cells or label released from dead cells could be detected in lymphoid tissue. Spleen cells were labelled and adjusted to a concentration of  $2.5 \times 10^7$  cells/ml. This suspension was divided into 2 aliquots. One was injected in 0.4 ml doses intravenously into 10 recipients. The other aliquot was frozen and thawed 4 times before being injected in 0.4 ml doses into a further 10 recipients. 5 recipients from each group were sacrificed 24 and 72 hrs after the intravenous injections. At 24 hrs the amount of radioactivity found in lymph nodes and spleens of recipients of dead cells was 5 and 10% of that found in mice which had received live cells. Significantly more activity was found in the lungs of recipients of dead cells than in the lungs of recipients of live cells, by 24 hrs. In both instances the majority of the counts found in the blood were present in the plasma. The mice<sup>were</sup> sacrificed at 72 hrs to ascertain if reutilisation had occurred with time.

Although the ratio of activity in the lymphoid tissue of recipients of live cells to that of recipients of dead cells had decreased, this was entirely due to a decrease in the counts present in the recipients of living cells. There was no increase in radioactivity in the lymph nodes or spleen of recipients of dead cells. The activity in the



lungs had also largely disappeared. Unexpectedly, there was no increase in the amount of activity recovered in the liver of mice injected with dead cells. The total recovery of counts was 40-50% using live cells and 30-40% with dead cells.

### 3. Localisation of allogeneic cells. Tables IV.6, IV.7 and IV.8.

The aim of this experiment was to determine whether lymphocytes killed in situ release label in a form that can be reutilised by previously unlabelled cells. It is assumed that the outcome of the host's response to the injection of allogeneic cells will be the death of the labelled cells. On the basis of this assumption, it would be predicted that, if there is no reutilisation of label, detectable radioactivity should eventually be eliminated.

$5 \times 10^6$  labelled CBA (H2-k) and C57Bl (H2-b) lymph node lymphocytes were injected separately into 15 syngeneic and 15 allogeneic recipients. Thus a four way transfer was accomplished. 5 mice in each group were killed 3, 5 and 11 days after the transfer. 9.4% and 5.3% of syngeneic cells were found in the lymph nodes and spleen of CBA mice at 3 days compared to 1.5 and 0.9% of allogeneic cells. Therefore only 16 and 17% as many cells were found in the allogeneic lymph nodes and spleen as were found in syngeneic lymph nodes and spleen. Similarly at 5 days the amount of radioactivity recovered from the lymph nodes and spleen of recipients of allogeneic cells was about 11 and 15% of that recovered in the syngeneic transfer. After 11 days, however, the difference was not so marked, but this was due to a decrease in the levels in the syngeneic tissues. The disappearance of label in

C57Bl recipients of allogeneic cells was not as pronounced as in the CBA recipients, especially in the spleen where the localisation of syngeneic cells was very low. There were no significant discrepancies in other organs between the groups.

The immune responses in these recipients would have included both host vs graft and graft vs host components, and the possibility that lymphocytes making a graft vs host response do not localise normally in mice cannot be excluded. The following experiment was therefore designed to investigate the localisation of semi-allogeneic cells which will be subjected to a host vs graft response.

#### 4. Localisation of semi-allogeneic lymphocytes. Table IV.9a and IV.9b.

The localisation of F<sub>1</sub> hybrid lymphocytes in normal and sensitised parental strain mice was investigated. Mice were immunised with 10<sup>8</sup> F<sub>1</sub> spleen cells, injected intraperitoneally 8 days before the transfer of labelled cells to produce an accelerated rejection of F<sub>1</sub> lymphocytes.

<sup>75</sup>Se-labelled (CBA x C57Bl) F<sub>1</sub> lymph node lymphocytes were injected into normal CBA and C57Bl mice, sensitised CBA and C57Bl mice and syngeneic F<sub>1</sub> mice. The mice were killed 24 hrs later. As can be seen from Table IV.9a, surprisingly less radioactivity was found in the lymph nodes and spleen of unsensitised CBA and C57Bl mice than was found in the equivalent tissues of F<sub>1</sub> mice. There was a considerably enhanced elimination of label in sensitised recipients, the CBA mice performing better than the C57Bl mice. Table IV.9b expresses the amount of radioactivity recovered in normal and sensitised recipients of semi-allogeneic cells as a percentage of the amount recovered from the

equivalent tissues of  $F_1$  recipients of  $F_1$  cells.

There was no significant difference between any of the groups in the distribution of label in any of the other organs. The total activity recovered was 40-50% in the allogeneic transfers compared to 64% in the syngeneic transfer.

### C. Studies on the immunocompetence of labelled cells

The immunocompetence of labelled cells was assessed by their ability to localise to antigen, proliferate in response to antigen and produce YM and YG antibody.

#### 1. Localisation and proliferation of labelled cells. Fig. IV.5.

In Chapter II it was demonstrated that the ability of lymphocytes to transfer the proliferative response to oxazolone as measured by uptake of  $^{125}\text{I}$ -UdR, 4 days after transfer into irradiated recipients, provides a sensitive assay of T cell function. In the present experiment advantage was taken of the fact that the gamma radiation from  $^{125}\text{I}$  and  $^{75}\text{Se}$  can be easily distinguished, and therefore organs containing both isotopes can be counted. Ten irradiated recipients were injected with  $2.5 \times 10^6$   $^{75}\text{Se}$  labelled lymph node cells and 10 with the same number of unlabelled lymph node cells. Half the mice in each group were painted with oxazolone and half with alcohol. Four days after the application of antigen the localisation of labelled cells in the draining and contralateral axillary and brachial nodes was measured by counting in the  $^{75}\text{Se}$  channel, and the proliferation of labelled cells was estimated by measuring the incorporation of  $^{125}\text{I}$ -UdR in the

same lymph nodes. No difference was found in the proliferative response of labelled and normal lymphocytes in either the draining, contralateral or alcohol stimulated nodes. An accumulation of labelled cells was found in the lymph nodes of recipients of oxazolone, where the counts in the draining-nodes derived from  $^{75}\text{Se}$  were 3 times those found in nodes of unstimulated mice.

2. Ability of labelled spleen cells to transfer a primary humoral response to sheep red cells. Fig. IV.6 and IV.7.

(a) Response of  $2 \times 10^7$  cells. Fig. IV.6

Ten irradiated recipients were given  $2 \times 10^7$   $^{75}\text{Se}$  labelled spleen cells intravenously and 10 were injected with  $2 \times 10^7$  normal spleen cells. The same day the mice received  $10^8$  sheep erythrocytes by the same route. Five and 7 days later the spleens of the irradiated recipients were plaqued against sheep erythrocytes and the number of direct and indirect plaque forming cells were determined. The results revealed no significant difference in the number of plaque forming cells present in the spleens from recipients of labelled or normal cells.  $\gamma\text{G}$  antibody producing cells were not detected until day 7. As a precaution to ensure that labelled cells did not lyse red cells non-specifically, control mice received saline in lieu of sheep erythrocytes. The spleens from these recipients produced about 60 plaque forming cells per spleen. Prior to plaquing, spleens were randomly counted for radioactivity and were found to contain about 16,000 cpm (that is, 8% of the injected dose).

(b) Response of  $10^7$ - $10^8$  cells. Fig. IV.7

The above experiment utilised one dose of spleen cells. It is possible that the presence of a small number of damaged cells in the labelled population may not be detected in this system.

Mice were lethally irradiated and repopulated with  $10^7$ ,  $3 \times 10^7$ ,  $6 \times 10^7$  or  $10^8$  labelled or normal spleen cells. One group of mice were lethally irradiated and were not injected with lymphocytes. The mice received  $10^8$  sheep red cells intravenously the same day. Seven days later the spleens of the recipients were plaqued against sheep red cells and the number of  $\gamma$ M and  $\gamma$ G producing cells were determined. Recipients of labelled and unlabelled cells produced similar dose response curves (Fig. IV.7). The number of plaque forming cells per spleen increased concomitantly with the number of cells that the recipients had received. Mice which had been irradiated and not repopulated failed to produce plaques to sheep erythrocytes.

D. Localisation of labelled cells from various sources

1. Lymph node lymphocytes. Table IV.10, Fig. IV.8.

$5 \times 10^6$  labelled lymph node cells were injected intravenously into 36 syngeneic recipients and groups of 6 were killed 6, 24 hrs and 2, 3, 4 and 5 days later. The percentage of the injected radioactivity which was found in various organs after 6 and 24 hrs is shown in Table IV.10. The decline in activity recovered from the lymph nodes, spleen, liver and blood over 5 days is shown in Fig. IV.8.

Table IV.10 shows that more activity was recovered from the lymph nodes 24 hours after injection of cells than at 6 hrs, and less activity was recovered from the spleen. The total amount of radioactivity recovered from the excised organs was 83.7% of the injected dose at 24 hrs and 73.7% at 6 hrs. Radioactivity in the lymph nodes and spleen declined over 5 days with levels in the spleen reaching 14% of their peak value which occurred at 6 hrs and 33% of their 24 hr. value. Levels in the lymph nodes did not fall as rapidly over 5 days, being 30% of their peak 24 hr value and 50% of their 6 hr value. Localisation in the liver remained at 16-20% throughout the period, and the radioactivity recovered from blood decreased from 10% at 6 hrs and 24 hrs to 7-8% over days 3, 4 and 5.

2. Cortisone resistant thymocytes. Table IV.11

$6 \times 10^6$  labelled cortisone resistant thymocytes were injected intravenously into 9 recipients, 6 were killed at 6 hrs and 3 at 24 hrs.

As can be seen from Table IV.11, the activity recovered from the lymph nodes did not change between 6 and 24 hrs, but the activity recovered from the spleen decreased during this period. It should be noted that there were only 3 mice sacrificed at 24 hrs and the variations imply that the values for lymphoid tissue and spleen may be unreliable. The total amount of radioactivity recovered was 70.7% of the injected dose at 6 hrs and 54% at 24 hrs.

3. Thymocytes. Table IV.12.

$5 \times 10^6$  labelled thymocytes were injected into 15 recipients, and groups of 5 were killed at 6, 24 and 120 hrs.

As can be seen from Table IV.12 the amount of radioactivity recovered from the lymphoid tissue and spleen was extremely low, 3.5 and 5.6% of the injected dose at 6 hrs. Indeed, the activity recovered from all organs counted was only 38.1% of the injected dose. By 24 hrs the amount of activity recovered declined, with levels in spleen and liver decreasing more rapidly than levels in the lymphoid tissue. The levels of activity in the lymph nodes remained constant between 6 and 24 hrs. At day 5 only 19.5% of the injected dose was recovered.

#### 4. Rat thoracic duct cells. Table IV.13

Thoracic duct cells are a more viable population than lymphocytes that have been teased away from an organ. Therefore the localisation in the spleen, liver, lymph nodes and blood was compared with the results obtained from mice. In previous experiments reported in this chapter, 4-10% of radioactivity was found in the blood; it was decided to perfuse one rat to remove the blood from all the tissues to ascertain whether and to what extent the blood content of an organ contributes to its radioactive counts.

$2.18 \times 10^8$  labelled rat thoracic duct cells in 3.5 mls were injected intravenously into 2 syngeneic rats weighing 250 gms. The rats were sacrificed 24 hrs later. One rat was perfused for 30 mins with phosphate buffered saline before removing any organs. Samples of various organs were removed, weighed, and counted for radioactivity. The results in Table IV.13 were expressed as the percentage of the injected dose/gm tissue or ml fluid and as the percentage of lymph node activity. Localisation in the liver was very low. The rat liver

weighs about 10 gm and the activity recovered represented about 3.8% of the injected dose. The localisation in the spleen was low compared to localisation of lymph node lymphocytes in the spleens of mice (Table IV.10). The spleen weighed 600 mg and the percentage of the injected dose that was recovered from the spleen was about 4%. The amount of activity recovered from the lymph nodes of the non-perfused rat was 14% of the injected dose/gm tissue, and from the perfused rat was 10% of the injected dose. The total lymph nodes in the rat weigh about 1 gm. The total blood contained about 4.9% of the injected dose, of which about 2.9% was found in the plasma, and 0.72% was associated with red blood cells. The counts found in the perfusate represented 3.48% of the injected dose, of which 2.36% were found in the supernatant and 0.81% in the cells of the perfusate.

##### 5. Localisation of $^{51}\text{Cr}$ labelled spleen cells. Table IV.14.

Previous experiments suggested that the localisation patterns of  $^{75}\text{Se}$  labelled lymphocytes and  $^{51}\text{Cr}$  labelled lymphocytes differ slightly, especially regarding localisation in the spleen. To confirm that this phenomenon was due to the label and not simply to those of different experimental conditions, the localisation of  $^{51}\text{Cr}$  labelled spleen cells was investigated and compared to the localisation of freeze/thawed spleen cells from the same cell suspension. The results are shown in Table IV.14. The localisation of live  $^{51}\text{Cr}$  spleen cells at 24 hrs are similar to the results reported by other workers (Mainbridge *et al.*, 1966; Zatz & Lance, 1970). The levels had declined by 72 hrs. It is interesting that no activity was detected in the thymus except in the case of dead cells at 72 hrs. The difference in the amount of activity



recovered from the lymph nodes and spleen of recipients of live and freeze/thawed cells were similar to that obtained when cells were labelled with  $^{75}\text{Se}$ . The amount of activity recovered from the spleen and lymph nodes of recipients of dead cells was considerably less than that recovered from the equivalent tissue of recipients of live cells. At 72 hrs, however, the discrepancy was not as marked. When  $^{75}\text{Se}$  labelled spleen cells were injected the activity of lymphoid tissue and spleen had decreased by 72 hrs in both recipients of live and dead cells. In this experiment, however, the activity decreased only in the recipients of live cells. It was observed in the experiment which utilised  $^{75}\text{Se}$  (Table IV.5) that there was no tendency for the label from dead cells to be found in the liver. This phenomenon was also observed in this experiment. In the present studies, however, significantly larger amounts of activity were recovered from the kidneys of recipients of dead cells than in the kidneys of recipients of live cells.

**Plate 1**

**CBA thymocytes labelled with  $^3\text{H}$ -methionine.**

**1a: a large thymocyte (14 $\mu$ ) with 20-50 grains.**

**1b: two medium sized thymocytes (9 $\mu$  and 6 $\mu$ ) with 20-50 grains and 3-20 grains respectively**

**1c: a large thymocyte (13 $\mu$ ) with 50+ grains**

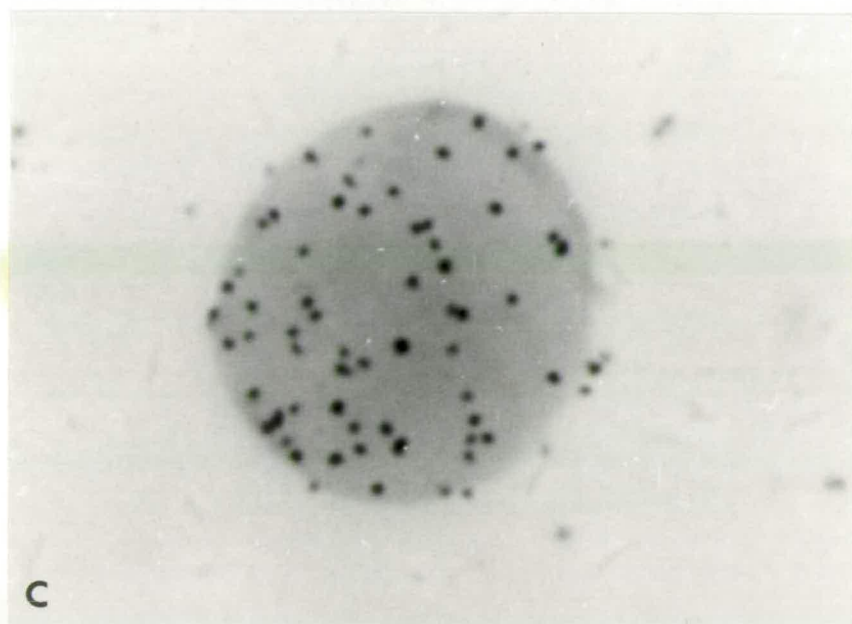
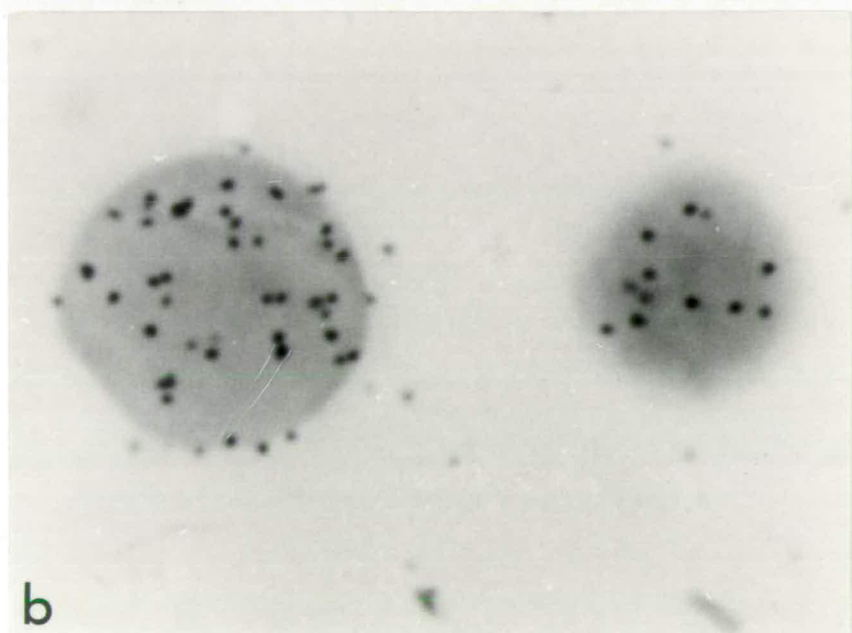
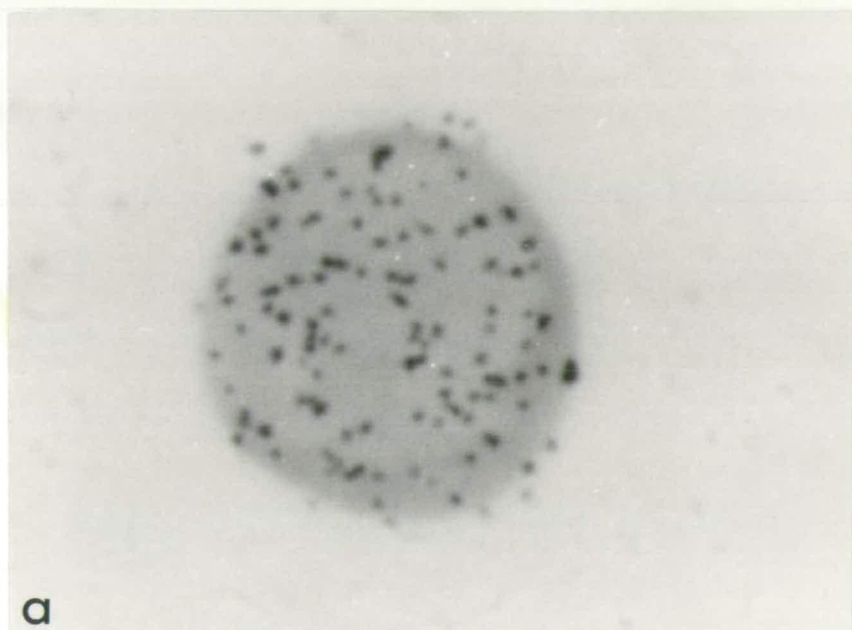
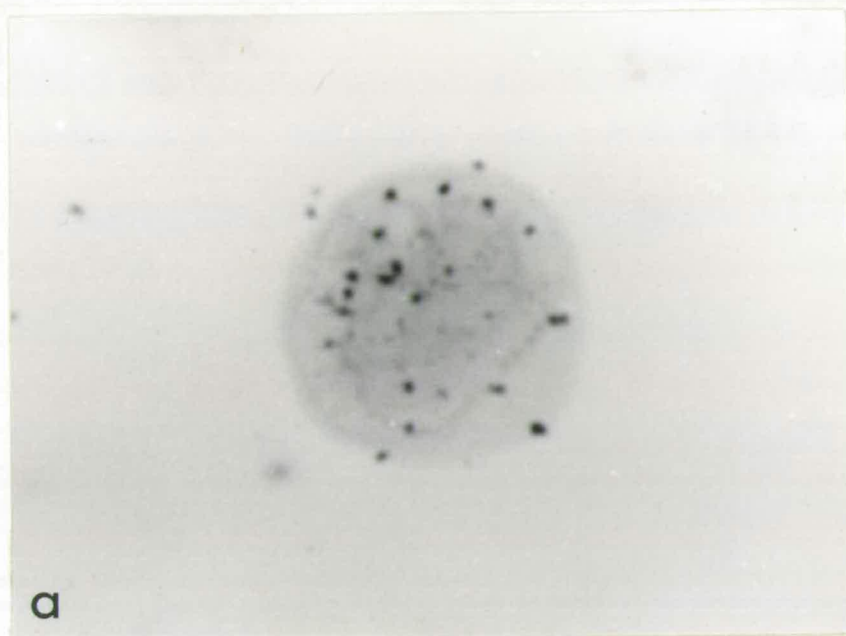


Plate 2

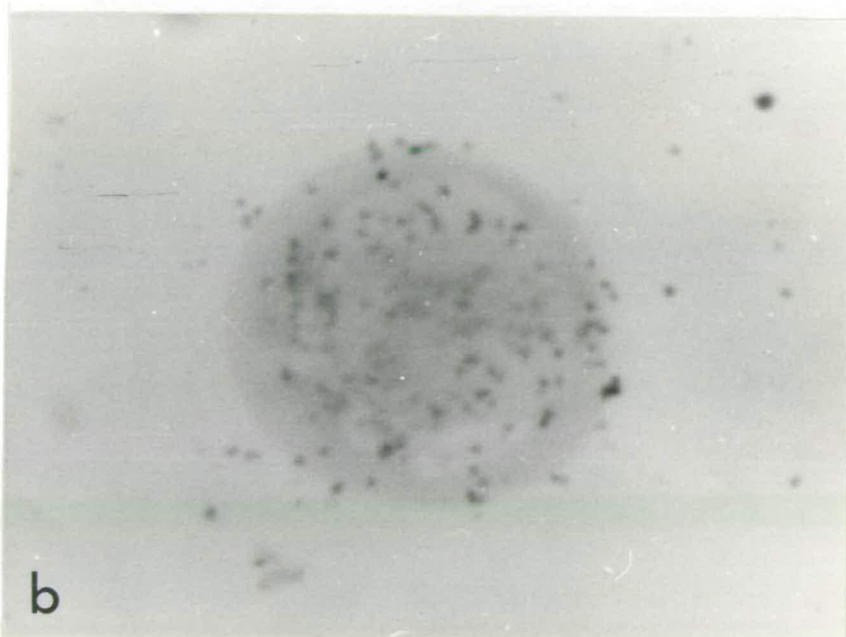
CBA lymph node lymphocytes labelled with  $^3\text{H}$ -methionine

2a: a medium sized lymphocyte (10 $\mu$ ) with 5-20 grains

2b: a large lymphocyte (15 $\mu$ ) with 50+ grains.



a



b



Plate 3

Nu/nu lymph node lymphocytes labelled with  $^3\text{H}$ -methionine.

3a: a large (14 $\mu$ ) and a medium sized lymphocyte (9 $\mu$ ) with 50+ and 5-20 grains respectively.

3b: two medium sized lymphocytes (10 $\mu$  and 8 $\mu$ ) with 20-50 and 5-20 grains respectively.

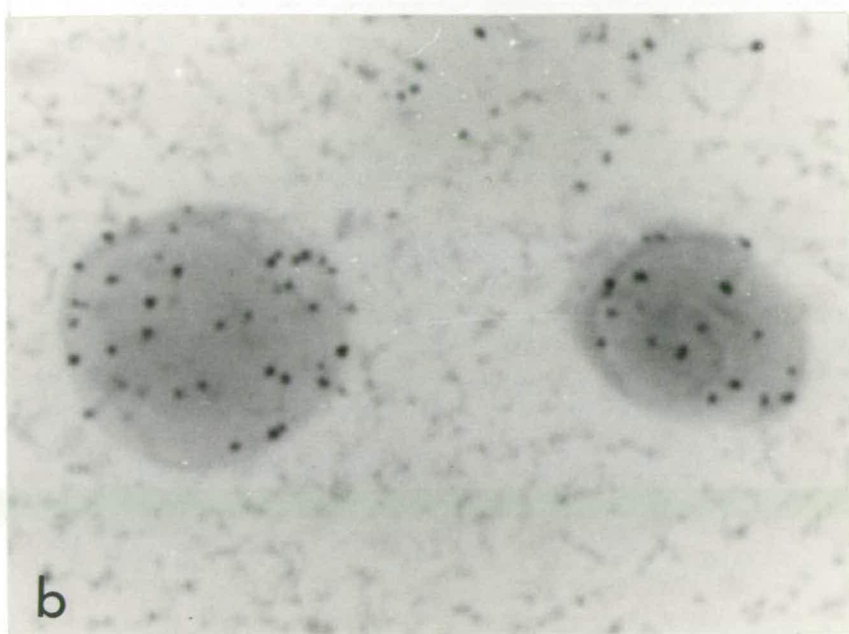
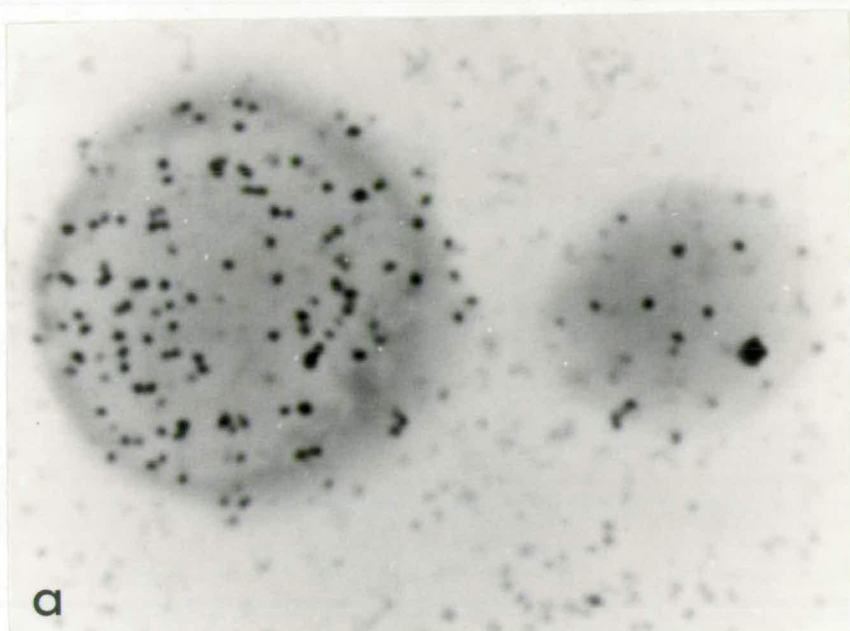




Fig. IV.1.

Incorporation of  $^{75}\text{Se}$  by CBA lymph node lymphocytes, cultured in vitro with  $1\mu\text{Ci}$ ,  $5\mu\text{Ci}$  and  $10\mu\text{Ci}/10^7$  cells, for 60 min. in Hanks BSS and 10% foetal calf serum and subsequently in the presence of 'cold' Medium 199.



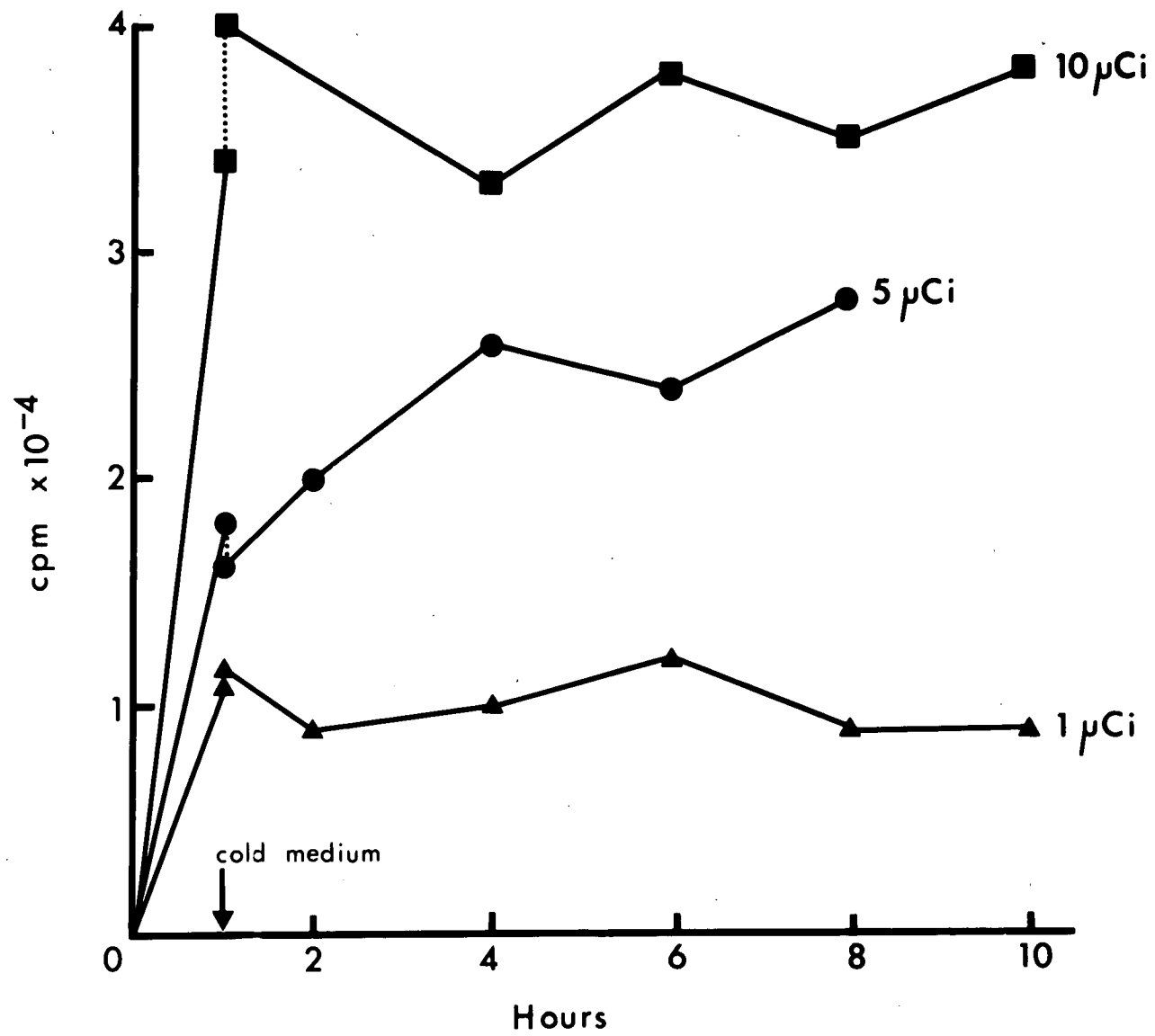


Fig. IV.2.

Incorporation of  $^{75}\text{Se}$  by CBA lymph node lymphocytes, cultured in vitro with  $2\mu\text{Ci}/10^7$  cells/ml for 60 mins in Hanks BSS and 10% foetal calf serum and subsequently in the presence of 'cold' Medium 199. Radioactivity found in  $10^7$  cells  $\blacktriangle$  and in the TCA precipitate from  $10^7$  cells  $\triangle$  .

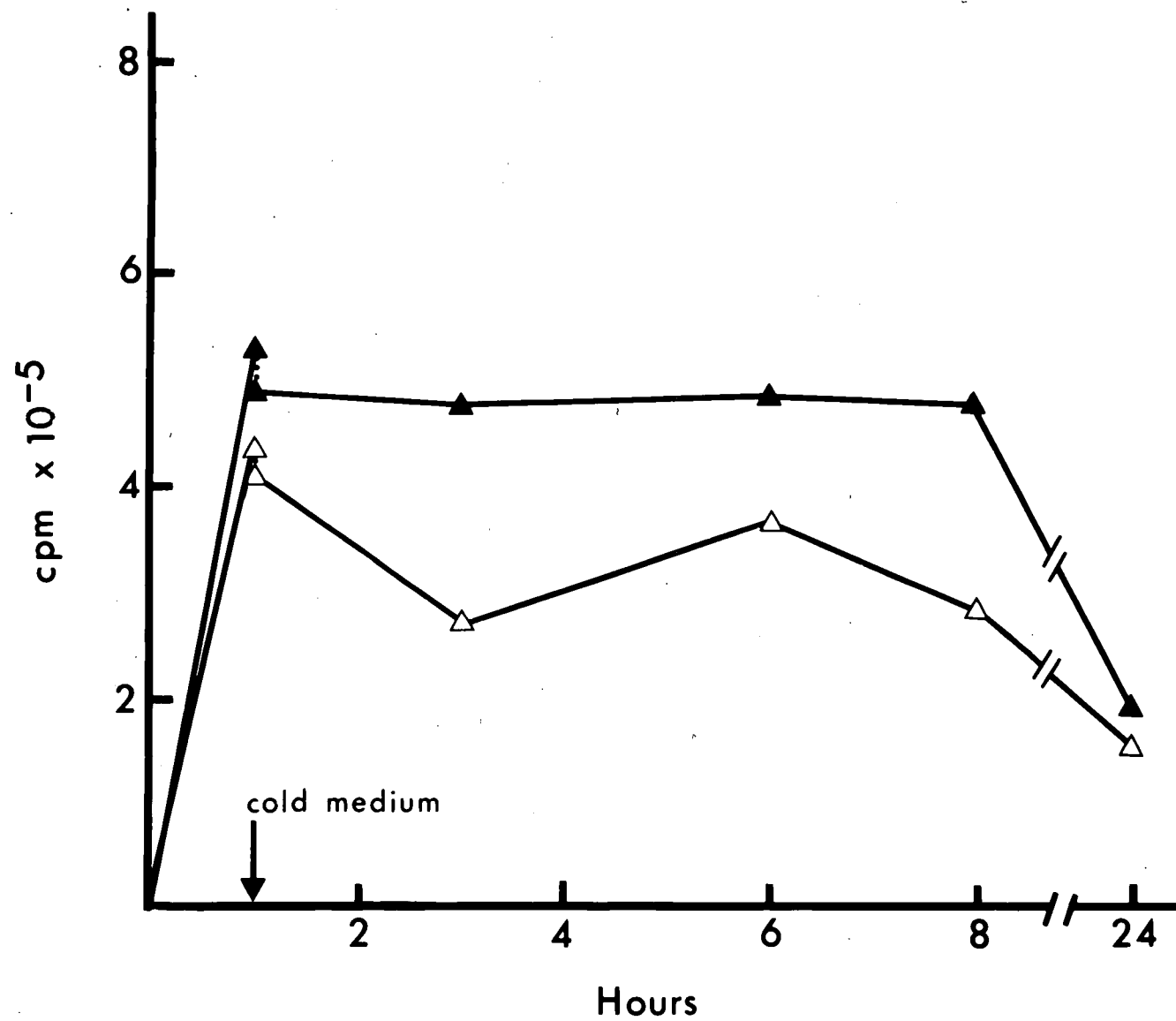
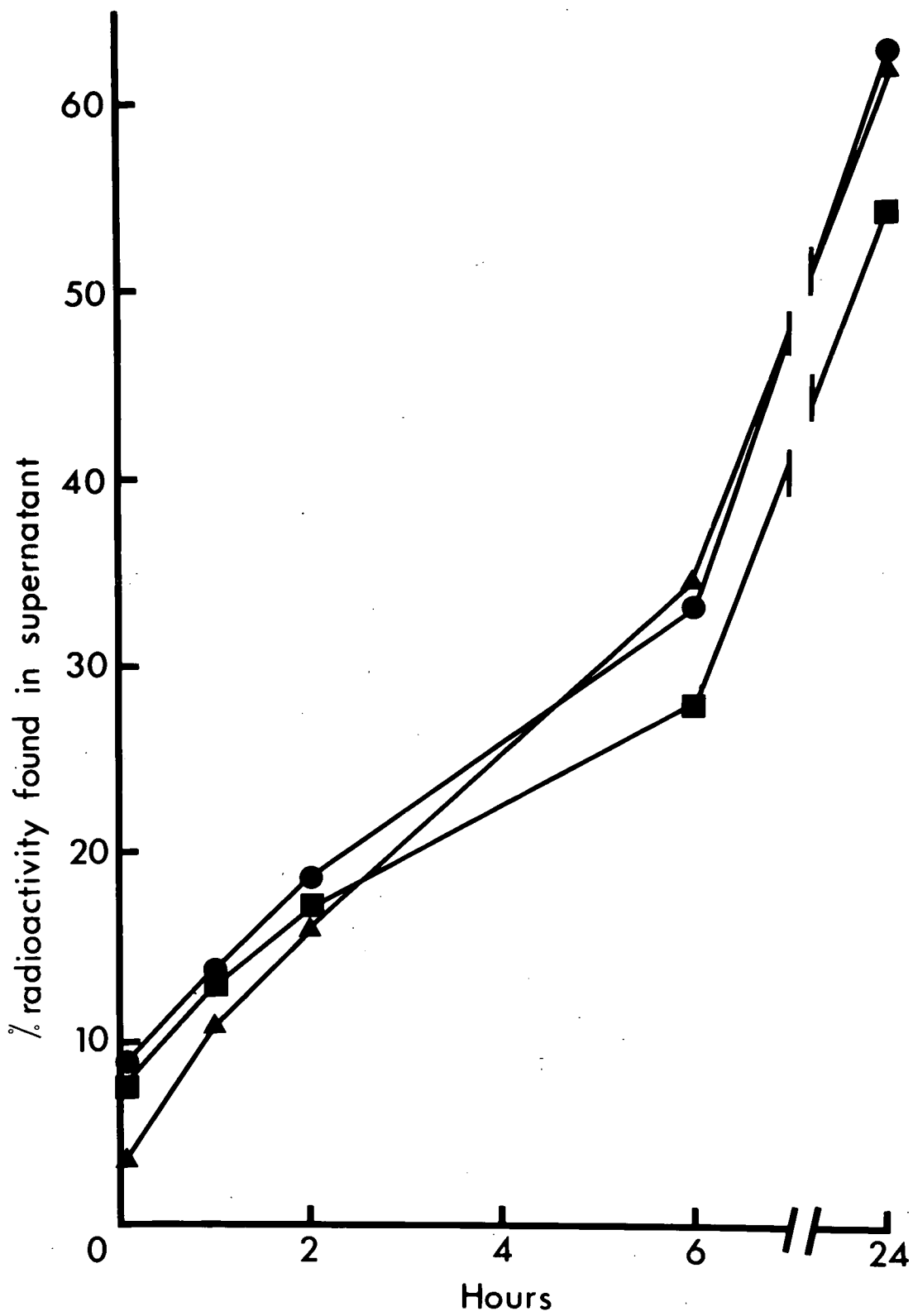





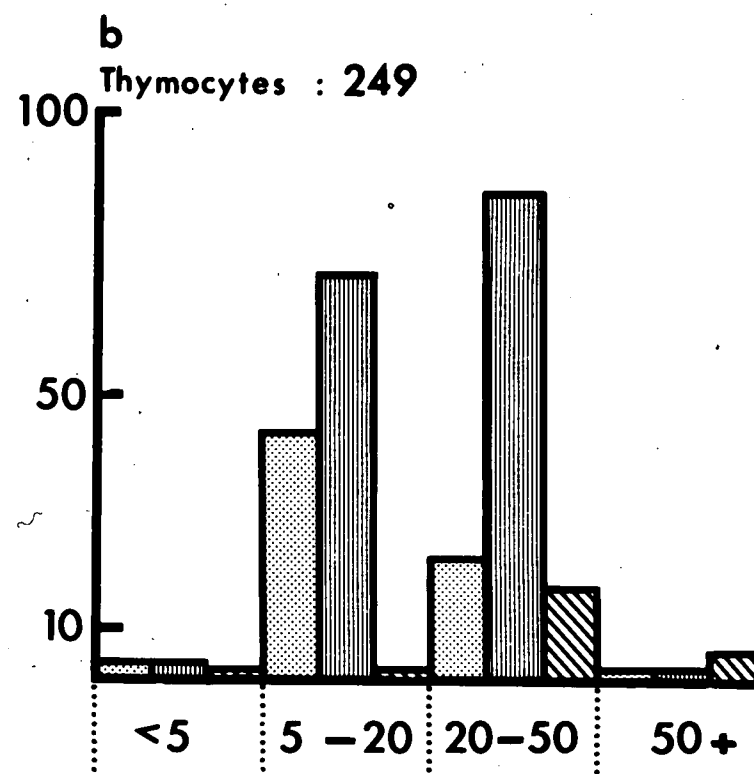
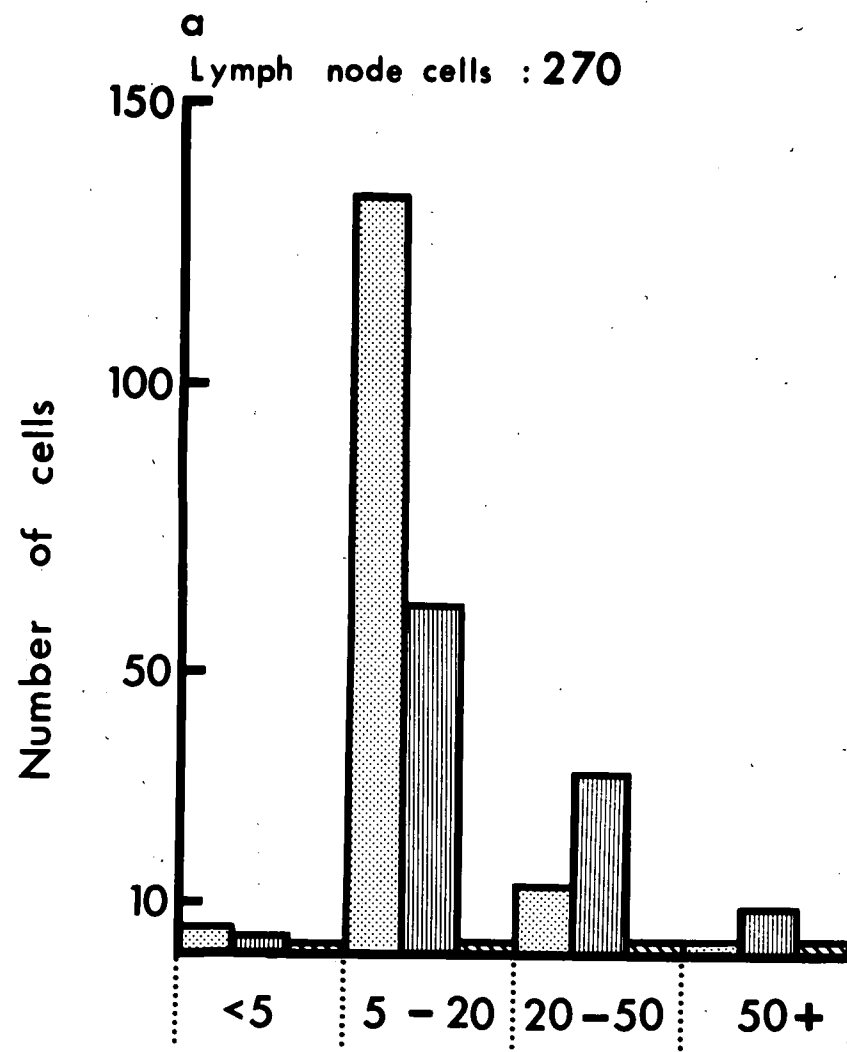
Fig. IV.3.

Release of  $^{75}\text{Se}$  from labelled rat thoracic duct lymphocytes,  
1, 2, 4, 6 and 24 hrs after being cultured in vitro at  
concentrations of  $10^5$  cells/ml ● ,  $10^7$  cells/ml ■ and  $5 \times 10^7$   
cells/ml ▲ .



**Figs. IV.4a, 4b and 4c.**

**Distribution of silver grains amongst  $^3\text{H}$ -methionine-labelled CBA lymph node lymphocytes (a), CBA thymocytes (b) and lymph node cells from the athymic nu/nu mouse (c). The number of small cells , medium sized cells  and large cells  containing <6, 5-20, 20-50 and 50+ grains is shown.**



Grain counts / cell

c  
nu / nu : 437

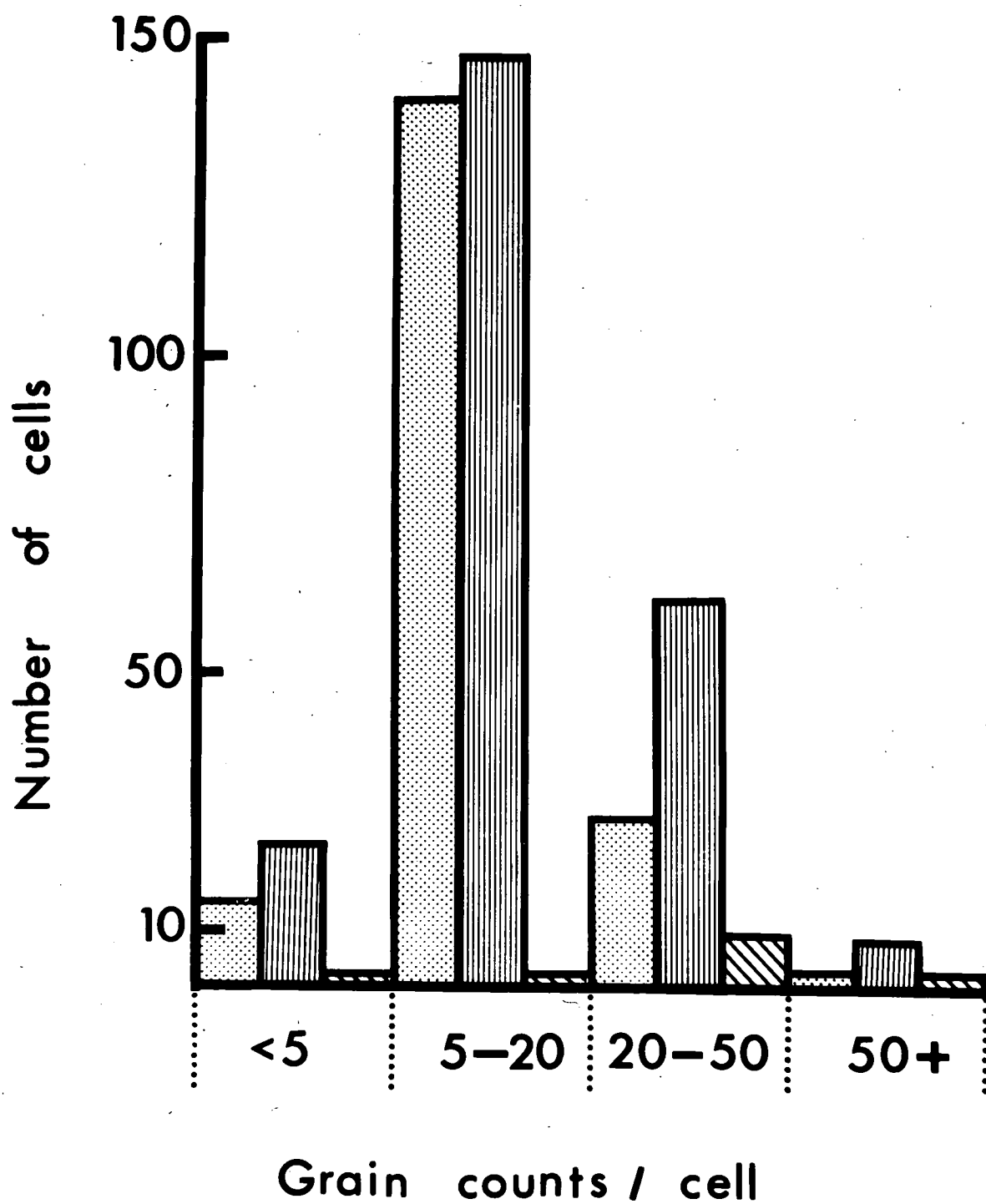








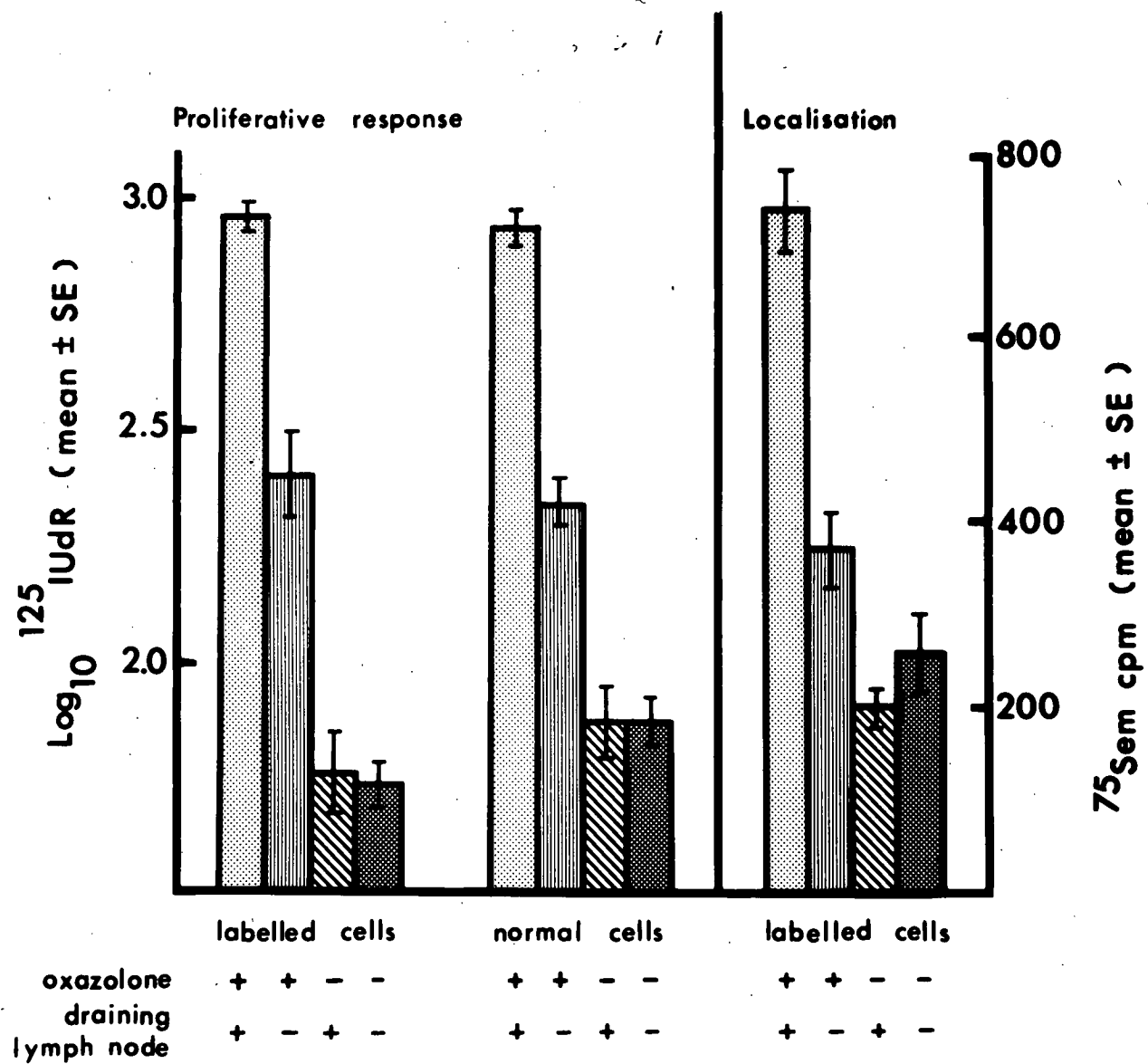






Fig. IV.5

Uptake of  $^{125}\text{I}$ UdR by the draining  and contralateral  axillary and brachial lymph nodes of irradiated (900R) recipients of oxazolone and the draining  and contralateral  lymph nodes of irradiated recipients of alcohol, 4 days after administration of antigen and 5 days after receiving  $2.5 \times 10^6$   $^{75}\text{Se}$ -labelled or normal lymph node cells intravenously. The  $^{75}\text{Se}$  content of the draining  and contralateral  axillary and brachial lymph nodes of recipients of oxazolone and the  $^{75}\text{Se}$  content of the draining  and contralateral  lymph nodes of irradiated recipients of alcohol, 5 days after receiving  $2.5 \times 10^6$   $^{75}\text{Se}$  labelled lymph node cells is shown.



**Fig. IV.6**

**PFC response to sheep erythrocytes of spleens from mice which had been irradiated (900R) and injected intravenously with  $2 \times 10^7$  normal or  $^{75}\text{Se}$ -labelled spleen cells and  $10^8$  sheep erythrocytes, measured 5 and 7 days after irradiation and injection of cells and antigen.   $\gamma$ MPFC;   $\gamma$ GPFC.**

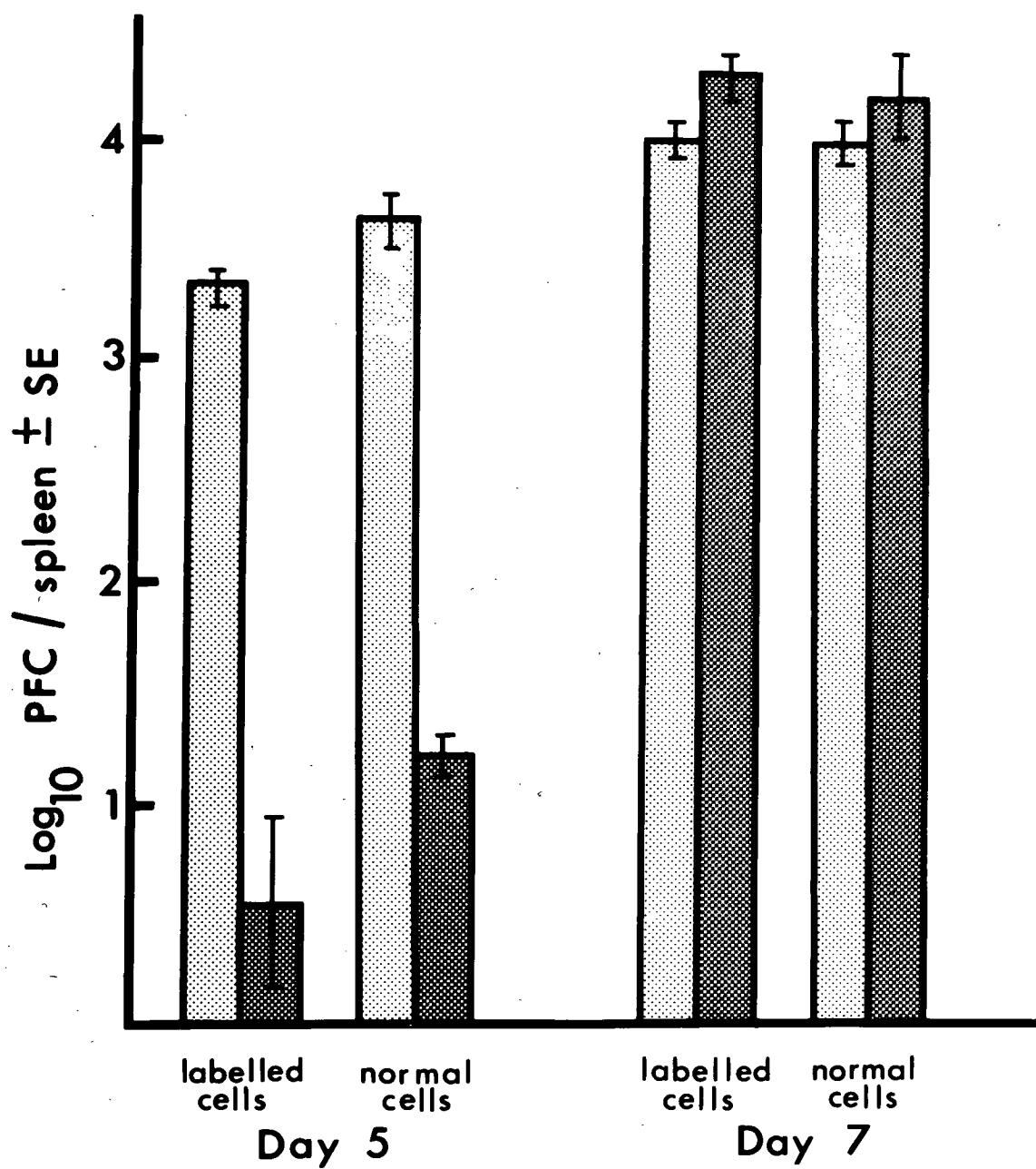


Fig. IV.7

PFC response to sheep erythrocytes of spleens from mice which had been irradiated (900R) and injected intravenously with  $10^7$ ,  $3 \times 10^7$ ,  $6 \times 10^7$  or  $10^8$  normal or  $^{75}\text{Se}$ -labelled spleen cells and  $10^8$  sheep erythrocytes, measured 7 days after irradiation and the injection of lymphocytes and antigen.



$\gamma\text{M}$  response from recipients of normal cells



$\gamma\text{M}$  response from recipients of labelled cells



$\gamma\text{G}$  response from recipients of normal cells



$\gamma\text{G}$  response from recipients of labelled cells

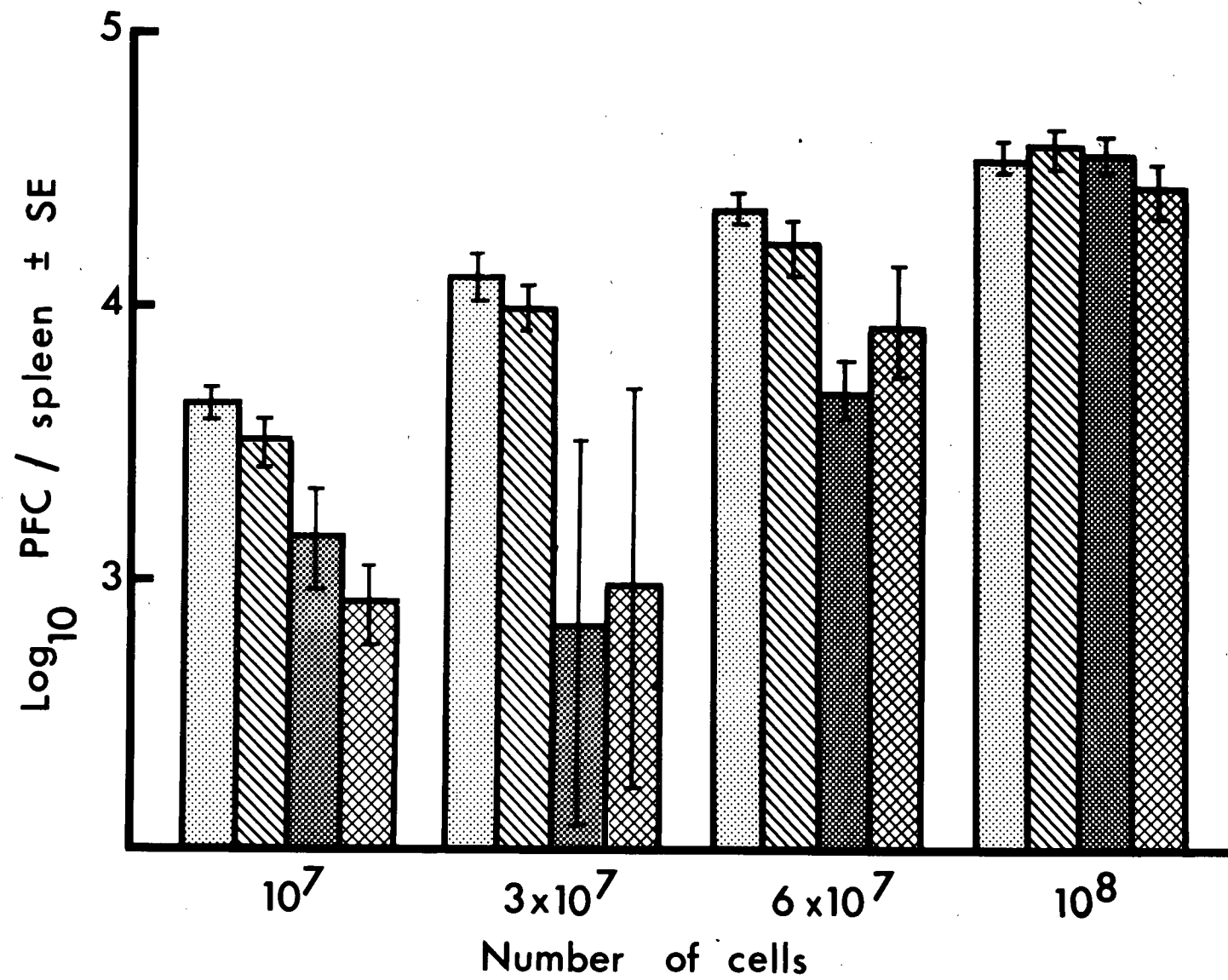


Fig. IV.8

Recovery of radioactivity, expressed as percentage of the injected dose in the spleen  $\blacktriangle$  , lymph nodes  $\bullet$  , liver  $\blacksquare$  and blood  $\blacklozenge$  of CBA mice 6 hrs and 1-5 days after the intravenous injection of  $5 \times 10^6$   $^{75}\text{Se}$ -labelled syngeneic lymph node cells.

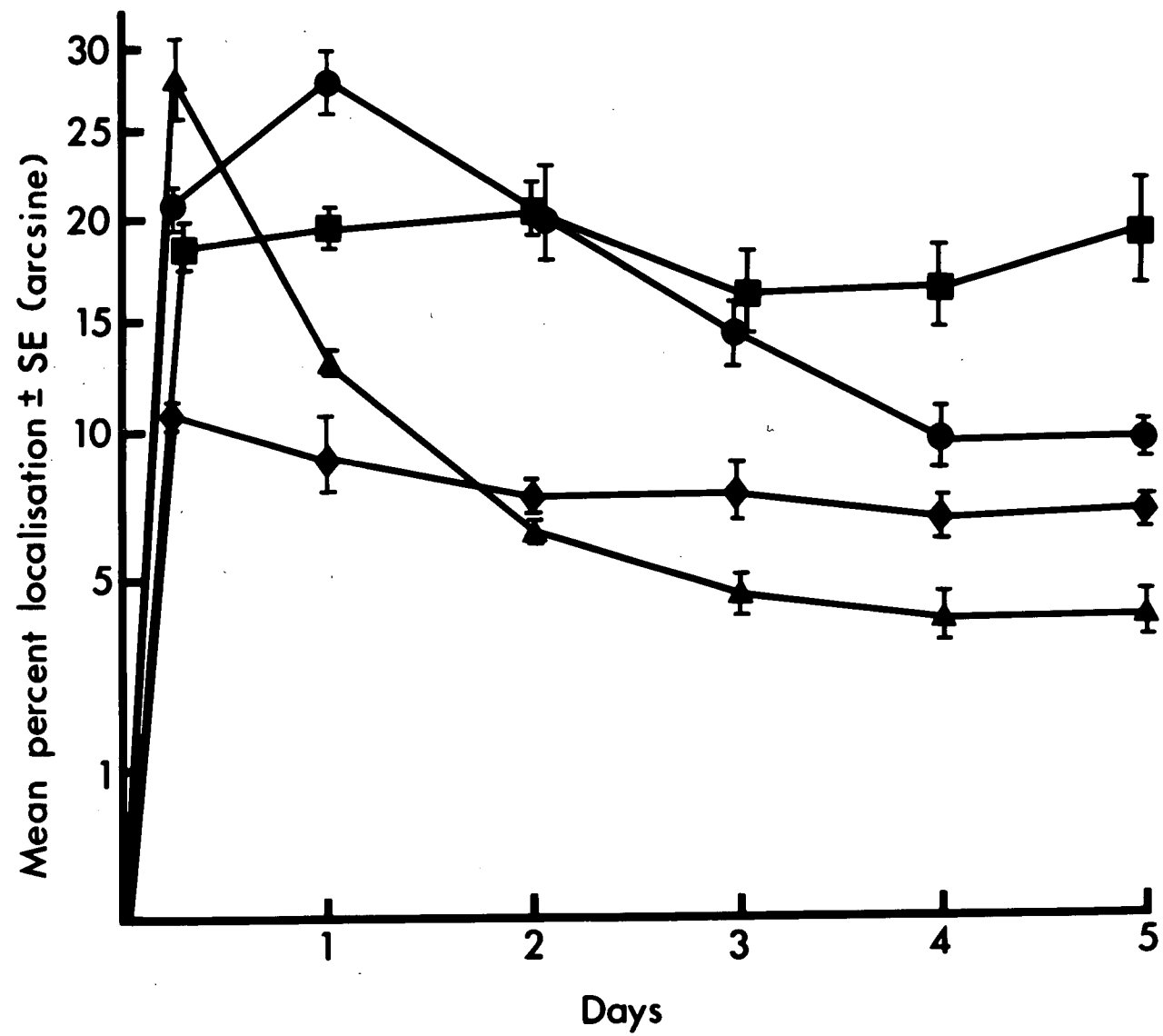




TABLE IV.1

Percentage of radioactivity in the supernatant of cultures  
containing  $5 \times 10^4$  <sup>75</sup>Se-labelled WF tumour cells in the  
presence and absence of  $10^7$  syngeneic lymph node cells

Culture	Time after initiation of culture (hours)				
	0	1	2	6	24
WF + LN	BG	BG	22.0	ND	33.0
WF + LN	11	BG	8.6	10.5	16.0
WF only	BG	18.0	6.0	25.0	33.0
WF only	BG	9.4	4.0	16.3	48.0

BG = background counts

WF = WF tumour cells

LN = lymph node cells

ND = Not determined

TABLE IV.2

## Incorporation of 75-selenomethionine

Details of lymphocytes				Details of 75-selenomethionine		
Exp.	type*	$\mu\text{Ci}/10^7$	$\text{cpm}/10^7$	Specific Activity	batch	$\text{CO}_2$
SeI	LNC	5	90,154	36.2mCi/mg	602	+
SeII	LNC	5	134,000	"	"	+
SeIII	LNC	5	43,460	"	"	-
SeIVT	LNC	5	96,000	"	"	+
SeIV	LNC	5	300,000	21 mCi/mg <sup>o</sup>	833	+
	LNC	5	200,000	"	"	+
SeV	LNC	2.5	160,000	"	"	+
SeVI	LNC	2	90,000	"	"	+
	Thy	2	161,000	"	"	+
	CRT	2	91,000	"	"	+
	Sp	2	90,000	"	"	+
	BM	2	130,000	"	"	+
SeVII	Thy	2	240,000	"	"	+
SeIX	LNC <sup>o</sup>	2	200,000	"	"	+
SeSBC	Sp	2	220,000	52.6mCi/mg	993	+
SeX	LNC <sup>o</sup>	2	ND	64.6mCi/mg	86	+
SeC	CRT	2	137,000	"	"	+
SeT	TDL**	2	240,000	68.6mCi/mg	386	+
SeXI	LNC	2	90,000	"	"	+

\* Unless otherwise stated the cells were obtained from CBA mice

<sup>o</sup> Cells obtained from (CBAX BIO) F<sub>1</sub> mice

\*\* RAT thoracic duct cells

LNC = lymph node cells; Thy = thymocytes; CRT = cortisone resistant thymocytes;

SP = spleen cells; BM = bone marrow cells.

TABLE IV.3

Distribution of grains amongst lymphocytes labelled  
with  $^3\text{H}$ -methionine

Source of cells	No. cells counted	Percentage of cells containing number of grains			
		< 5	5-20	20-50	50+
Lymph nodes	270	2.96	74.10	18.52	3.33
nu/nu lymph nodes	437	8.60	66.59	22.88	2.06
Thymocytes	249	2.4	45.78	50.20	2.80

TABLE IV.4

Distribution of radioactivity in CBA mice 2 and 24 hours after the intravenous injection of  $5 \times 10^6$  75-Sem labelled lymph node cells containing 35,520 cpm, or supernatant from labelled cells containing 3,470 cpm

Percentage of administered dose in various organs									
Time (hours)	Inoculum	Total Lymph Nodes	Spleen	Liver	Femur (x2)	Lung	Thymus	Blood (1ml)	Total
2	cells mean	14.2	16.1	14.9	1.7	3.7	0.1	3.8	54.3
	SD	3.8	3.5	0.8	0.7	0.8	0.07	1.4	
2	Sup* mean	3.2	4.4	12.5	0.5	0.4	1.2	4.9	27.2
	SD	1.4	0.3	2.0	0.4	0.4	1.2	0.4	
24	cells mean	20.4	8.8	12.5	0.76	1.4	0.2	3.7	47.7
	SD	4.4	0.1	1.4	0.2	0.1	0.02	0.9	
24	sup* mean	2.9	1.3	9.5	1.1	0.4	0.3	3.0	18.5
	SD	1.6	0.4	1.7	1.3	0.5	0.5	1.9	

\*sup = supernatant

SD = standard deviation

TABLE IV.5

Distribution of radioactivity in CBA mice 24 and 72 hours after the intravenous injection of  $10^7$  75-Sem-labelled live and freeze-thawed syngeneic spleen cells

Percentage of administered dose in various organs												
Time (hours)	Inoculum	Total lymph nodes	Spleen	Liver	Femur (x2)	Lung	Thymus	Blood (1ml) cells	plasma	Gut	Kidney (x2)	Total
24	Live											
	mean	9.6	8.3	10.8	0.82	1.1	0.16	0.7	2.2	14.0	4.1	52.0
	SD	1.3	1.0	0.5	0.2	0.3	0.02	0.4	0.8	3.9	0.3	
	Freeze-thawed											
	mean	0.5	0.8	11.3	0.5	6.7	0.1	0.9	2.7	11.8	4.9	40.1
	SD	0.1	0.2	0.7	0.04	2.0	0.02		0.3	1.8	0.5	
72	Live											
	mean	4.1	3.5	13.2	0.6	0.7	0.2	3.0	2.1	9.3	3.3	39.9
	SD	2.8		1.1	0.02	0.05	0.06	0.1	0.2	2.1	1.2	
	Freeze-thawed											
	mean	0.3	0.6	12.4	0.4	1.2	0.1	1.1	1.7	8.3	3.8	29.8
	SD	0.09	0.07	1.0	0.03	0.1	0.1	0.2	0.3	0.9		

SD = Standard deviation.

TABLE IV.6

Distribution of radioactivity in CBA and C57B1, B10 mice three days after the intravenous injection of  $5 \times 10^6$  <sup>75</sup>-Sem labelled allogeneic or syngeneic lymph node cells

Donor strain	Recipient strain	Percentage of administered dose in various organs						
		Total lymph nodes	Spleen	Liver	Femur (x2)	Lung	Thymus	Blood (1ml)
CBA	CBA mean	9.4	5.3	12.7	0.3	0.9	0.2	3.9
	SD	0.6	0.5	1.0	0.1	0.2	0.2	0.7
B10	CBA mean	1.5	0.9	12.5	0.3	0.5	0.1	2.7
	SD	0.2	0.1	0.6	0.1	0.1	-	0.9
B10	B10 mean	7.7	2.5	11.1	0.2	0.7	0.1	2.9
	SD	1.8	0.6	2.1	-	0.2	-	0.4
CBA	B10 mean	1.8	1.0	13.9	0.2	0.5	0.1	2.4
	SD	0.4	0.1	0.7	-	-	-	0.5

SD = Standard deviation

TABLE IV.7

Distribution of radioactivity in CBA and C57Bl, B10 mice  
five days after the intravenous injection of  $5 \times 10^6$  <sup>75</sup>-Sem labelled  
allogeneic or syngeneic lymph node cells

Donor strain	Recipient strain	Percentage of administered dose in various organs						
		Total lymph nodes	Spleen	Liver	Femur (x2)	Lung	Thymus	Blood (1ml)
CBA	CBA mean	6.4	2.7	11.9	0.2	0.7	0.1	2.7
	SD	1.3	0.6	1.2	0.04	0.1	0.02	0.1
B10	CBA mean	0.7	0.4	10.4	0.2	0.4	0.1	2.9
	SD	0.1	0.1	0.5	0.1	0.02	0.02	0.9
B10	B10 mean	4.3	1.8	12.9	0.2	0.5	0.1	2.3
	SD	0.4	0.2	0.8	0.03	0.03	0.01	0.1
CBA	B10 mean	1.1	0.6	12.9	0.2	0.5	0.1	2.2
	SD	0.2	0.1	0.6	0.02	0.04	0.02	0.1

SD = Standard deviation

**TABLE IV.8**

**Distribution of radioactivity in CBA and C57Bl, B10 mice  
eleven days after the intravenous injection of  $5 \times 10^6$  75-Sem labelled  
allogeneic or syngeneic lymph node cells**

Donor strain	Recipient strain	Percentage of administered dose in various organs						
		Total lymph nodes	Spleen	Liver	Femur (x2)	Lung	Thymus	Blood (1ml)
CBA	CBA mean	1.6	0.7	7.2	0.2	0.4	0.1	2.5
	SD	0.06	0.1	0.4	0.01	0.04	0.02	0.6
B10	CBA mean	0.8	0.4	8.3	0.2	0.4	0.1	ND
	SD	0.2	0.1	1.5	0.04	0.04	0.02	ND
B10	B10 mean	1.7	0.8	9.5	0.4	0.4	0.1	1.7
	SD	0.6	0.2	3.6	0.6	0.1	0.02	0.5
CBA	B10 mean	0.6	0.3	7.8	0.1	0.4	0.1	1.4
	SD	0.06	0.03	0.3	0.03	0.02	0.01	0.2

**SD = Standard deviation**



**TABLE IV.9a**

Distribution of radioactivity in sensitised\* and normal CBA and C57B1 mice and in (CBAX X C57B1) F<sub>1</sub> mice 24 hours after the intravenous injection of  $2.7 \times 10^6$  75-labelled (CBA X C57B1) F<sub>1</sub> lymph node lymphocytes

Recipient strain	Sensitised	Percentage of administered dose in various organs							
		Total lymph nodes	Spleen	Liver	Femur (x2)	Lung	Thymus	Blood (1ml)	Total
CBA	-	mean	5.9	6.9	27.2	1.2	1.5	0.5	52.6
		SD	1.9	1.2	2.2	0.3	0.5	0.1	0.5
CBA	+	mean	1.16	2.0	28.4	1.3	1.8	0.6	43.8
		SD	0.4	1.0	4.6	0.3	0.2	0.2	1.1
F <sub>1</sub>		mean	19.1	11.2	24.0	1.1	1.7	0.6	64.0
		SD	2.7	2.9	3.1	0.7	0.9	0.4	1.1
C57B1	+	mean	2.8	2.8	30.0	1.8	1.7	0.6	48.6
		SD	0.9	0.9	5.4	0.8	0.4	0.1	0.8
C57B1	-	mean	4.1	4.3	27.6	1.5	1.7	0.7	45.7
		SD	2.3	1.5	4.6	0.2	0.4	0.2	0.8

\* CBA and C57B1 mice which had received  $10^8$  (CBA x C57B1) F<sub>1</sub> spleen cells i.p. eight days prior to the injection of labelled cells

SD = Standard deviation

TABLE IV.9b

Amount of radioactivity in lymphoid tissue of recipients of semi-allogeneic lymphocytes expressed as a percentage of the amount of activity recovered from the equivalent tissue of  $F_1$  recipients of  $F_1$  cells 24 hours after the injection of  $^{75}\text{Se}$  labelled cells

Donor	Recipient	% activity in tissue*	
		Total lymph nodes	Spleen
$F_1$	Normal CBA	31	62
$F_1$	Sensitised CBA	6	18
$F_1$	Normal C57Bl	31	38
$F_1$	Sensitised C57Bl	15	25

$$* \% \text{ activity} = \left[ \frac{\text{activity in recipients of semi-allogeneic cells}}{\text{activity in } F_1 \text{ recipients of } F_1 \text{ cells}} \right] \times 100$$

TABLE IV.10

Distribution of radioactivity in CBA mice 6 and 24 hours after  
the intravenous injection of  $5 \times 10^6$  <sup>75</sup>-Sem labelled syngeneic  
lymph node cells

Time (hours)	Percentage of administered dose in various organs							
	Total lymph nodes	Spleen	Liver	Femur (x2)	Lung	Thymus	Blood (1ml)	Total
6 mean	20.7	28.5	19.4	2.2	2.4	0.2	10.6	83.7
SD	2.8	5.7	3.8	0.2	0.6	0.05	0.8	
24 mean	28.4	12.9	19.6	1.0	2.8	0.3	8.9	73.7
SD	4.6	1.7	1.9	0.2	1.4	0.05	0.2	

SD = Standard deviation

TABLE IV.11

Distribution of radioactivity in CBA mice 6 and 24 hours after  
the intravenous injection of  $6 \times 10^6$  75-Sem labelled syngeneic  
cortisons resistant thymocytes

Percentage of administered dose in various organs										
Time (hours)		Total lymph nodes	Spleen	Liver	Femur (x2)	Lung	Thymus	Blood (1ml)		Total
								cells	plasma	
6	mean	11.5	21.9	21.8	2.6	2.8	0.2	3.0	8.2	70.7
	SD	1.7	1.2	3.1	0.4	0.6		1.1	1.0	
24*	mean	11.7	8.5	22.6	1.0	1.8	0.2	4.2	4.4	54.0
	SD	3.1	3.1	3.9	0.1	0.4	0.05	1.5	1.5	

SD = Standard deviation

\* three mice only in this group

TABLE IV.12

Distribution of radioactivity in CBA mice 6, 24 and 120 hours  
after the intravenous injection of  $5 \times 10^6$  75-Sem labelled  
syngeneic thymocytes

Time (hours)	Percentage of administered dose in various organs						
	Total lymph nodes	Spleen	Liver	Blood	Femur (x2)	Lung	Total recovery
6 mean	3.5	5.6	18.3	6.7	1.8	2.2	38.1
SD	0.3	0.4	2.3	1.0	0.1	0.3	
24 mean	3.7	2.3	13.2	5.3	0.6	0.7	25.8
SD	0.4	0.4	1.7	0.8	0.1	0.2	
120 mean	2.6	0.7	11.6	3.6	0.4	0.6	19.5
SD	0.2	0.1	2.2	6.6	0.04	0.2	

SD = Standard deviation

TABLE IV.13

Distribution of radioactivity in AO rats 24 hours after the  
intravenous injection of  $2.1 \times 10^8$  75-Sem labelled syngeneic  
thoracic duct cells

Tissue	Non-perfused		Perfused	
	percent inj. dose /gm or ml	percent LN act.	percent inj. dose /gm or ml	percent LN act.
Liver	0.45	2.67	0.38	3.78
Spleen	7.17	42.27	7.86	77.60
L.Kid.	0.58	3.43	0.39	3.86
R.Kid.	0.60	3.54	0.39	3.86
Gut	0.96	5.64	0.83	8.15
Testis	0.16	0.94	0.14	1.36
Epid.	0.07	0.44	0.06	0.57
Abdo.Sk.	0.17	0.99	0.08	0.75
FP.Sk.	0.15	0.86	0.09	0.90
Muscle	0.08	0.46	0.06	0.63
Lung	0.94	5.52	0.37	3.69
Thymus	0.25	1.47	0.19	1.84
LN	16.96	100	10.13	100
Sal, Gl.	0.21	1.22	0.09	0.91
Brain	0.07	0.41	0.06	0.59
Tail	0.20	1.15	0.08	0.82
Bone	0.22	1.31	0.17	1.68
Blood	0.29/ml, 4.5% total		0.33/ml, 4.9% total	
Plasma	0.37/ml, 3.3% total		0.33/ml, 2.9% total	
Blood, WBC	0.11/ml, 0.67% total			
Blood, RBC	0.15/ml, 0.92% total			

L.Kid. = left kidney; R.Kid. = right kidney; Epid. = Epididimis;  
Abdo.Sk = Abdominal skin; FP.Sk = Foot Pad skin; LN = Lymph Nodes;  
Sal. Gl = Salivary glands.

TABLE IV.14

Distribution of radioactivity in CBA mice 24 and 72 hours  
after the intravenous injection of  $10^7$   $^{51}\text{Cr}$  labelled live or  
freeze-thawed syngeneic spleen cells

Time (hours)	Inoculum	Percentage of administered dose in various organs											
		Total lymph nodes	Spleen	Liver	Femur (x2)	Lung	Thymus	blood(1ml) Cells    Plasma		Gut	Kidney (x2)	Total recovery	
24	Live	mean	18.8	22.1	17.1	1.5	4.9	0.0	0.2	0.2	4.5	0.8	70.2
		SD	3.2	2.2	3.5	0.2	1.4	0.0	0.08	0.05	0.8	0.2	
24	F/T*	mean	1.2	1.9	10.5	0.6	5.0	0.0	0.1	0.7	1.0	6.5	27.1
		SD	0.7	0.4	5.1	0.2	2.8	0.0	0.03	0.2	0.2	0.1	
72	Live	mean	15.1	17.3	19.6	0.9	3.8	0.0	0.1	0.02	3.0	0.6	60.3
		SD	2.9	1.8	3.0	0.2	1.5	0.0	0.05	0.02	0.7	0.1	
72	F/T	mean	1.5	3.1	15.7	0.5	2.1	0.2	0.1	0.1	0.7	5.6	29.7
		SD	2.3	2.4	2.3	0.2	0.7	0.3	0.1	0.1	0.7	2.0	

\*FT = Freeze/Thawed      SD = Standard deviation

TABLE IV.15

Distribution of radioactivity found in perfused rats 24 hours after the intravenous injection of thoracic duct cells labelled with different isotopes

Results are expressed as per-cent injected dose/gm tissue or /ml fluid

Tissue	$^3\text{H}$ Uridine(5)*	$^{14}\text{C}$ Uridine(2)	$^{75}\text{Se}$ m(1)	$^3\text{H}$ leucine(1)	$^{14}\text{C}$ leucine(1)
Liver	0.37 (0.24-0.49) <sup>x</sup>	0.58 (0.58-0.58)	0.38	0.22	0.56
Spleen	16.34 (11.07-25.07)	21.08 (16.0-26.0)	7.86	12.30	16.95
L.K.	0.42 (0.34-0.56)	0.29 (0.23-0.35)	0.39	0.49	0.31
R.K.	0.41 (0.32-0.60)	0.27 (0.23-0.31)	0.39	0.46	0.28
Gut	0.58 (0.35-0.92)	0.54 (0.45-0.63)	0.83	1.41	0.38
Testis	0.23 (0.09-0.37)	0.03 (0.03-0.03)	0.14	0.24	0.11
Epid	0.056(0.05-0.07)	0.03 ( " " )	0.06	0.15	0.05
Abdo.sk.	0.13 (0.05-0.22)	0.04 (0.03-0.05)	0.08	0.18	0.14
F.P. sk	0.084(0.02-0.17)	0.05 (0.04-0.06)	0.09	0.29	0.16
Muscle	0.124(0.07-0.22)	0.02 (0.02-0.02)	0.06	0.17	0.07
Lung	0.72 (0.41-0.95)	1.05 (0.93-1.12)	0.37	1.23	1.85
Thymus	0.24 (0.11-0.33)	0.27 (0.26-0.28)	0.19	0.62	0.31
LN	15.47 (11.75-20.21)	11.84 (10.3-13.3)	10.13	19.23	13.43
Sal.Gl.	0.27(0.17-0.36)	0.13 (0.12-0.15)	0.09	0.37	0.14
Brain	0.08 (0.02-0.24)	0.02 (0.02-0.02)	0.06	0.15	0.14
Blood, tot	4.72 (3.61-6.17)	1.68 (1.35-2.01)	4.9	5.5	3.94
Plasma "	2.50 (2.31-2.70)	0.52 (0.35-0.68)	2.9	6.0	1.98
Perfusate	23.4 (14.6-33.0)	2.81 (2.34-3.28)	3.48	32.14	ND
Sup.Perfus	24.4 (18.9-29.9)	1.85 (1.7-2.00)	2.36	ND	0.67

\* the number in brackets indicates the number of animals

<sup>x</sup> numbers in brackets indicate the lowest and the highest amount of activity recovered from that organ.



## DISCUSSION

### 1. The isotope

<sup>75</sup>Se-selenomethionine is an analogue of methionine containing selenium. The isotope of selenium, <sup>75</sup>Se-selenium has a half life of 120 days. It decays by 100% electron capture to stable arsenic emitting a number of rays, the majority of which (55.5% and 58.6%) have energies of 0.136 and 0.265 MeV.

Clinically, <sup>75</sup>Se-selenomethionine has been used for detecting diseases of the pancreas (Blau and Bender, 1962), malfunction of the parathyroid gland (Potchen, 1967), and detection of malignant lymphomas (Herrera et al., 1968). However the long biological half life of <sup>75</sup>Se-selenomethionine and its facile placental transmission do not render it an ideal isotope for clinical use (Sodee et al., 1965). Experimentally, it has been used to study the regulation of platelet production in rodents (Harker, 1970; Amorosi et al., 1971; Najean & Ardaillou, 1969; Evatt & Levin, 1969; Penington, 1969), and the rate of turnover of human erythrocytes (Penner, 1966).

The question of whether the metabolic pathways which utilise selenomethionine and methionine are identical has not been unequivocally established. The data appear to be best interpreted by the suggestion that the short term utilisation of the two amino acids is very similar. In the longer term however there may be differences in the utilisation and catabolism of the products containing the isotopes. The time course of incorporation of the two amino acids labelled with <sup>14</sup>C into liver protein

phosphatidyl-choline and RNA was found to be very similar (Pan *et al.*, 1964), as was their ability to be activated to the adenosyl amino acid and act as methyl donors (Bremer & Natori, 1960).  $^{75}\text{Se}$ -selenomethionine was incorporated by hens into egg white protein. The isotope was located in the same part of the peptide chain as  $^{35}\text{S}$ -methionine (Armando & Gitter, 1968), but differences in metabolism were apparent by the failure to detect  $^{75}\text{Se}$ -selenocysteine in egg white protein. While the turnover of muscle protein was approximately similar when assessed using either  $^{75}\text{Se}$ -selenomethionine or  $^3\text{H}$ -lysine, the turnover rate of serum and liver proteins appeared abnormally low with  $^{75}\text{Se}$ -selenomethionine due to recycling of the compound (Waterlow *et al.*, 1968). The uptake of radioactivity in rats injected with  $^{75}\text{Se}$ -selenomethionine could be prevented by a simultaneous injection of cold selenomethionine, but not by methionine, therefore (Holland *et al.*, 1966) suggesting the presence of separate metabolic pathways.

After the injection of the pure isotope into an animal, whole body autoradiographs suggest that incorporation most rapidly occurs in those organs which possess high rates of protein turnover, the pancreas and the liver (Hansson & Jacobsson, 1966).

## 2. The labelling technique

The procedure which was developed using  $^{75}\text{Se}$ -selenomethionine ( $^{75}\text{Sem}$ ) appears to be a reliable and reproducible method of labelling various lymphocyte populations. On average,  $10^7$  cells when incubated with  $2\mu\text{Ci}$  of  $^{75}\text{Sem}$  incorporated 100,000-200,000 cpm. This represents only about 2% of the counts to which the cells were exposed, and it may explain

why after 3 washings, 10% of the total count could be found in the supernatant. The labelling medium consisted of a balanced salt solution enriched with foetal calf serum: complete medium was not used on the assumption that the presence of methionine would inhibit the uptake of  $^{75}\text{Se}$ . An experiment to test this assumption was not performed, but the fact that Gowans (1962) labelled rat lymphocytes with  $^3\text{H}$ -leucine in M199 shows that it is possible to label cells with a radioactive amino acid in a complete medium. Fig. IV.1 shows that incorporation of counts did not increase proportionately with the numbers of  $\mu\text{Ci}$  presented to the cells. It is possible that this effect may be due to an absolute limit on the rate of incorporation. This preliminary experiment suggested that  $2\mu\text{Ci}$  or less per  $10^7$  cells would provide sufficient incorporation of activity for the purposes of most experiments.

### 3. Incorporation of $^{75}\text{Se}$ by different cell populations

About 75% of the isotope which was incorporated into lymphocytes was recovered in the TCA precipitate (Fig. IV.2). This figure of 75% is given credence by the finding of Penington (1969) that 75% of the isotope in labelled platelets could be precipitated by perchloric acid. The location of the remaining 25% is unclear, but it may be present as the free amino acid.

The most accurate way of investigating the distribution of label among different lymphocyte populations is by autoradiography. The obvious isotope to have used in this experiment would have been selenomethionine labelled with either  $^{14}\text{C}$  or  $^3\text{H}$ . These compounds were not commercially available, therefore  $^3\text{H}$ -methionine had to be used. An objection to this

experiment is that the incorporation of  $^3\text{H}$ -methionine by lymphocytes may be regulated by different factors from those that affect the incorporation of  $^{75}\text{Se}$ . In view of the evidence which suggests that the short term metabolism of the two compounds is similar (Foopan et al., 1964; Bremer & Natori, 1964; Armando & Gitter, 1968) and in the absence of autoradiographic data using selenomethionine, it is expedient (though not necessarily valid) to extrapolate from the results using  $^3\text{H}$ -methionine.

If a non-uniform distribution of grains amongst the lymphocyte population was found it could be due to differential incorporation by T and B cells, or differential incorporation by cells of different sizes or different metabolic rates. The fact that over 90% of lymph node lymphocytes from CBA mice, lymph node lymphocyte from nu/nu mice and thymocytes were labelled suggests that both T and B cells incorporate the isotope. Figs. IV.4a, b and c show the distribution of grains amongst small, medium and large lymphocytes and suggest a direct relationship between cell size and grain count. This accounts for the observation (Table IV. 2) that cells which were removed from tissues with high mitotic indices, that is, bone marrow and thymus cells, incorporated more isotope (in terms of cpm/ $10^7$  cells) than did lymphocytes from the lymph nodes or spleen.

#### 4. Elution of label

One of the major problems of using radioisotopes to label lymphocytes is the possible elution of label from cells in vivo, the subsequent fate of that label and the manner in which it affects the results.

Elution of label from cells probably reflects the rate of turnover of the compound into which the label was incorporated. Isotopes which provide the most stable labels, with the possible exception of Technitium 99m (Barth & Gillespie, 1974) are therefore those which are incorporated into cellular components with low turnover rates, such as DNA (Davidson, 1965). The present findings demonstrate that  $^{75}\text{Se}$  is not particularly stable within a lymphocyte and 50-55% is lost over 24 hrs (Fig. IV.3). This is equivalent to the rate of elution of isotope from  $^{51}\text{Cr}$  labelled lymphocytes (Rona, 1969) and  $^3\text{H}$ -uridine labelled lymphocytes (Goldschneider & McGregor, 1968b). The demonstration of elution in vitro may not however accurately reflect the situation in vivo. Fig. IV.3 shows that the rate of elution in vitro was not concentration dependent between  $10^5$  -  $5 \times 10^7$  cells.

The fact that isotope elutes from cells labelled with  $^3\text{H}$ -uridine and  $^3\text{H}$ -leucine in vivo is suggested by the finding that 23.4 and 32.14% of the injected dose was found in the perfusate of rats 24 hrs after the injection of labelled thoracic duct cells (G. Rannie, unpublished results). The equivalent value found from  $^{75}\text{Se}$  was only 3.4%. This could be either because the eluate was excreted, or because it was incorporated into a rapidly metabolising tissue, such as intestinal epithelium or pancreas. It appears unlikely that eluted  $^{75}\text{Se}$  is substantially reutilised by lymph node cells in view of the results derived from the reutilisation studies. Another aspect of the problem of elution is that if an isotope is unequally distributed among lymphocytes, the degree of elution may also vary. Non-uniform loss of label from lymphocytes over a 24 hr period could result in a population disproportionately represented by the cells that can be detected.

The possibility that  $^{75}\text{Se}$  might be a suitable isotope for use in cytotoxicity assays was investigated by labelling rat WF tumour cells and measuring the non-specific release of label. Unfortunately the  $5 \times 10^4$  tumour cells that were labelled contained too little radioactivity to assess accurately the amount of label released. Nevertheless it appears probable that 30-50% of the label was released over 24 hrs. This value is not inferior to that obtained for the elution of  $^{51}\text{Cr}$  from similar cells (67% over 24 hrs, P. Chisholm, pers. comm.), but it compares unfavourably with the claims that using  $^{125}\text{I}$ UdR only 4% of the label is released over 24 hrs (P. Chisholm, pers. comm.) and that Technitium 99m is completely stable (Barth & Gillespie, 1974).

#### 5. Reutilisation studies

Experiments were designed to encompass several situations which could result in host cells, previously unlabelled, becoming labelled. The source of label may be:

- a. Label in the supernatant. After 3 washes the supernatant may contain 10% of the total count in the injected dose. This is an overestimate, as the injected cells are resuspended in fresh medium.
- b. Label released from dead cells. Most cell suspensions contain a proportion of dead cells, some of the label may be washed away during preparation, but some will remain attached to the cells. Label which is slowly released from dead cells or cell debris may contribute to the radioactivity of host tissues.
- c. Label which elutes. As much as 50% of the activity of a cell suspension may be lost over 24 hrs.

- d. Label may be released from fragile cells (such as blasts) if they are damaged during the injection procedure.
- e. Label which is released from lymphocytes if they are killed within the lymphoid tissue as a consequence of an immune response.

It can be seen (Table IV.4) that the contribution made by activity in the supernatant to the activity which was found in lymph nodes and spleen was very small, approximately 0.29 and 0.15% of the lymph node and spleen activity could be attributed to supernatant. Most of the counts in the supernatant were excreted, although some might have been found in the intestine (as was found in Table IV.5).

The amount of activity that dead cells can contribute to lymph nodes and spleen was investigated (Table IV.5). At 24 hrs about 20 times and 10 times more radioactivity was found in the lymph nodes and spleen of recipients of live cells than in the recipients of dead cells. A similar difference was found when  $^{51}\text{Cr}$  was used as the label, in these studies (Table IV.14) and those of Bainbridge et al. (1966). Bainbridge et al. (1966) also injected heat killed (CBA x A) F1 cells into CBA recipients and found 120 times as much activity in the lymph nodes of recipients of live cells as recipients of dead cells. In this experiment a considerable amount of the activity from heat killed cells was recovered from the liver, a phenomenon not observed with  $^{75}\text{Se}$  frozen/thawed cells (Table IV.5) or  $^{51}\text{Cr}$  frozen/thawed cells (Table IV.14, and reported by Bainbridge et al., 1966). It is interesting that relatively high activity was recovered from the intestine although there was no difference in the intestinal recovery from live or dead cells. This and the fact

that  $^{75}\text{Se}$  may be rapidly incorporated in intestinal epithelium (Hansson & Jacobsson, 1966) suggests that the intestinal activity owes little to the localisation of living cells. Localisation in the lungs and excretion probably accounts for the number of dead cells that were not found in the lymph nodes and spleen. After 72 hrs there was a decline in the amount of activity which was found in the lymph nodes and spleens of all recipients, although this decline was more pronounced in the recipients of live than of dead cells. This suggests that the activity remaining at 72 hrs, 0.3-0.6% of the injected dose, will not disappear rapidly, and represents stably incorporated isotope, possibly in host cells. A similar conclusion may be drawn concerning results obtained using  $^{51}\text{Cr}$  (Table IV.14).

Studies on the localisation of allogeneic cells (Tables IV.6,7 and 8 and semi-allogeneic cells (Table IV.9) were designed to investigate the fate of label released from cells killed within the lymphoid tissue. A poor recovery of radioactivity from allogeneic recipients was found. The fact that the levels of recoverable activity fell between days 3 and 5 but remained constant between days 5 and 11 suggests that a low level of activity, about 0.8-0.4% of the injected dose may be incorporated into some low-turnover protein. These results cannot however be interpreted simply. It was anticipated that 3 days after the transfer of labelled cells, there would be no overt sign of the progressing immune response and that localisation in all groups would be equal. Any subsequent decrease in activity could be attributed to the death of the cells and elimination of their label. However, because no observations were made before 72 hrs, it is possible that an unknown proportion of the difference in the



recovery in lymph nodes and spleen between the allogeneic and syngeneic transfer may have been due to a failure of allogeneic cells to localise.

It was reasoned that the localisation of  $F_1$  cells in parental recipients would be simpler to interpret, because the response would be restricted to a host vs graft response. This experiment (Tables IV.9a and IV.9b) has not however overcome the objection that semi-allogeneic cells may not localise normally. However, sensitisation of the parental mice with  $F_1$  cells resulted in a considerable decrease in the amount of activity recovered from the lymph nodes and spleen of parental recipients. This decrease must have been due to death of the labelled cells and elimination of label. It is interesting that sensitisation of CBA mice proved more effective in eliciting immune rejection of cells than sensitisation of the C57Bl mice. This may be due to the deployment of different immune mechanisms, for example in the contribution made by humoral and cellular immunity. These results are similar to those of Bainbridge et al., (1966) who found 7.77% and 14.4% of  $^{51}\text{Cr}$  labelled (CBA x A) $_{F_1}$  cells were recovered in the lymph node and spleen of A mice compared to 1.54% and 9.26% of  $F_1$  cells found in the equivalent tissues of sensitised mice.

The interpretation of this experiment, in terms of the amount of re-utilisation, is hindered by the curiously low amount of activity that was recovered from the lymphoid tissue of unsensitised recipients of  $F_1$  cells. This suggests that either semi-allogeneic cells do not localise normally, or that they were subjected to a rapid host vs graft response that resulted in their disappearance. Parental rat cells have been found to localise in the lymphoid tissue and spleen of  $F_1$  hybrid recipients as efficiently as syngeneic cells (W.L. Ford, pers. comm.). It is therefore

reasonable to assume that  $F_1$  mouse cells localise normally in parental tissue. Lymphocytes have been found in lymph nodes within 15 mins of their injection (Gowans & Knight, 1964) and it may be possible that within 24 hrs they are killed by some immune mechanism. On the basis that the localisation of semi-allogeneic cells between these strains is normal, these results show that 1.2 and 2% of label from cells killed in situ is reutilised in the lymph nodes and spleen respectively of CBA mice. These figures probably overestimate reutilisation, because they may also reflect activity from cells that survived the immune response, and cell debris remaining in the lymphoid tissue. It is apparent that contribution of activity by the blood will be proportionately more in the lymph nodes and spleen of the parental strain recipients than in the equivalent tissues of  $F_1$  recipients.

Neither of the allogeneic transfer experiments are ideal models of reutilisation in vivo. A more satisfactory experimental system would be one that allowed normal localisation to occur followed by a treatment that would destroy the labelled lymphocytes.

To summarise the results from the reutilisation studies, 2.9% and 1.3% of the supernatant dose, 0.5% and 0.8% of the frozen/thawed cells and 1.16% and 2.0% of the total label released by cells killed in situ can be found in lymph nodes and spleen of CBA mice. What bearing do these results have to a more normal situation where viability is over 90% and cells are not subjected to a host vs graft response? A typical injected dose contains for example, 50,000 cpm. If this dose contained (at a maximum) 5,000 cpm in the supernatant, then the supernatant could

contribute 150 cpm throughout the lymph nodes and 50 cpm to the spleen. On the assumption that 10% of the cell inoculum was dead, this would represent 5,000 cpm which would distribute 25 cpm (0.5%) among the lymphoid tissue and 40 cpm (0.8%) to the spleen. The fate of the label which elutes from live cells at a rate of 50% over a 24 hr period may be explained by the supernatant, dead cells or reutilisation models. Because the label has been incorporated, the latter two models are probably more representative. Consider the example in which 1.16% of the label is reutilised in the lymph nodes and 2.0% in the spleen. Because 25,000 cpm (50% of the dose) is being slowly eluted over 24 hrs it may not be correct to assume that 1.16% of it, that is 250 cpm, and 2% of it, that is 500 cpm, is found among the lymph nodes and spleen respectively. Nevertheless, at some risk of overestimating the reutilisation, this would result in levels of about 425 cpm in the lymph nodes and 590 cpm in the spleen which do not represent the original inoculum of labelled, living cells.

#### 6. Immunocompetence of labelled cells

There are several ways in which <sup>75</sup>Se could impair the immunocompetence of labelled lymphocytes:

- a) By the deleterious effects of internal gamma radiation.
- b) If the label was incorporated into cell surface proteins, the selenium moiety could interfere with the functioning of membrane receptors.
- c) In the long term selenomethionine may not be metabolised in the same way as methionine (Hannsson & Jacobsson, 1966). This could lead to metabolic defects that would interfere with the synthesis of many proteins. Any disturbance in antibody formation or in the synthesis of surface proteins that participate in antigen receptors or in receptors

necessary for cell circulation could alter the immune behaviour of lymphocytes.

In the following experiments some parameters of immunocompetence were investigated; the ability of labelled cells to localise to antigen, to proliferate in response to antigen, and to produce humoral antibody.

The ability of lymphocytes to proliferate in response to oxazolone, as measured by the incorporation of  $^{125}\text{IUdR}$ , was demonstrated in Chapter II to be a sensitive assay of the presence of T cells. The results in Fig. IV.5 show that after 5 days in vivo  $2.5 \times 10^6$  labelled cells were not impaired in their ability to specifically recognise oxazolone and proliferate to the same extent as unlabelled cells.  $2.5 \times 10^6$  lymph node cells were estimated from the dose response curve in Chapter II (Fig. II.10) to be a suitably sensitive dose where any deviation in the amount of  $^{125}\text{IUdR}$  incorporation, due to damaged T cells, would be detected. Within 12 hrs of antigen administration, cell traffic through a draining node is retarded and this effect lasts 2 to 3 days (Ford, 1969; Zatz & Lance, 1971a; 1971b; Emeson & Thursh, 1971; Ford, 1972; Thursh & Emeson, 1972; Frost & Lance, 1974a). Considerably more counts from  $^{75}\text{Sem}$  were found in the nodes draining the site of oxazolone application than were found in the contralateral nodes, providing evidence of an accumulation of labelled cells in the antigen-stimulated node.

The ability of labelled spleen cells to recognise sheep red cells and produce humoral antibody was tested and the results are shown in Figs. IV.6 and IV.7. After 5 and 7 days in vivo (Fig. IV.6) the number of

direct ( $\gamma$ M) antibody and indirect ( $\gamma$ G) antibody producing cells produced by  $2 \times 10^7$  labelled spleen cells was equivalent to those produced by normal spleen cells. This implies that a critical number of labelled T and B cells were able to home to the spleen of the irradiated recipient, and these remained functional as regards their ability to cooperate, differentiate, and produce antibody. The fact that a 2 or 3 cell interactive process is necessary to achieve  $\gamma$ G production means that the kinetics of the cellular interactions of labelled cells might not have been identical to those of normal cells, even though the same end was achieved. The results in Fig. IV.7 however suggest probable identity between the labelled and the non-labelled spleen population, because of the similarity between the responses of a variety of doses of labelled and normal cells. Thus providing that B cells are the limiting factor in the transfer of the haemolysin response,  $^{75}\text{Se}$  did not affect the ability of spleen cells to migrate to the spleen in irradiated recipients, to recognise sheep erythrocytes and produce the necessary interactions with other cell types to produce antibody.

#### 7. Localisation of labelled cells from various sources

The localisation patterns of cells from various sources were examined in order to compare the results with those obtained using other isotopes, and to discover whether localisation conforms to that which would be predicted on the basis of their known immunocompetence and circulating properties.

The localisation of lymph node lymphocytes, cortisone resistant thymocytes, thymocytes and spleen cells is compared to results obtained by investigators who used  $^{51}\text{Cr}$  labelled cells. It is difficult to correlate results using  $^{75}\text{Se}$  with data using tritium labelled compounds, as the latter are nearly always expressed as autoradiographic data.

(a) Localisation of cells in lymph nodes and spleen

According to Zatz and Lance (1970) the pooled axillary, brachial, inguinal and mesenteric nodes represent about half the mass of total lymphoid tissue. This seems reasonable in view of the missing lumbar, renal, caudal, popliteal, parathyroid and auricular nodes. Accordingly in the present studies the results which are presented as 'total lymph nodes' were twice the counts obtained from the pooled axillary, brachial inguinal and mesenteric nodes.

(1) Lymph nodes

The localisation of  $^{75}\text{Se}$  labelled lymphocytes, from various sources, within the lymph nodes, 24 hrs after their injection correlates with the results that have been obtained using  $^{51}\text{Cr}$  labelled cells (Mainbridge *et al.*, 1966; Zatz & Lance, 1970; Blomgren & Andersson, 1972) at least within a range of variation to be expected from different experimenters.

The results conform to localisation patterns predicted on the basis of functional activities. The cells that were found to accumulate to the greatest extent in lymph nodes were lymph node lymphocytes, followed by cortisone resistant thymocytes, spleen cells and thymocytes. It has been estimated that 60% of lymph node lymphocytes are recirculating

(Zatz & Lance, 1970) and it is probable that these consist mainly of  $T_2$  (Cantor & Asofsky, 1970) and some  $B_2$  cells (Strober & Dilley, 1973a, 1973b). The number of lymph node seeking cells in a lymph node suspension is enriched by passaging them through an intermediate recipient and collecting the recipient's lymph node cells (Zatz & Lance, 1970). This probably eliminates non-recirculating short-lived T and B cells and spleen seeking cells. If 60% of lymph node cells are recirculating this might represent the expected recovery from lymph nodes and spleen, however it can be seen from Table IV.10 that actual recovery was less than this. This is probably due to a number of factors such as damage to cells during homogenisation or intravenous injection, or loss of label due to elution.

Cortisone resistant thymocytes exhibit some immunocompetence (Blomgren & Andersson, 1969; Blomgren & Andersson, 1970; Andersson & Blomgren, 1970; Cohen et al., 1970; Blomgren & Svedmyr, 1971; Mosier & Cantor, 1971) but lack the ability to synergise with spleen cells in a graft vs host response. This suggests they are not as mature as lymph node lymphocytes (Tigelaar & Asofsky, 1973). There is evidence that thymocytes which have recently migrated from the thymus require a period of maturation in the periphery before becoming mature recirculating cells (Mosier & Cantor, 1971; Cantor & Mosier, 1972). The fact that cortisone resistant thymocytes were found at levels of 11.7% in lymph nodes and 8.5% in the spleen confirms that they are able to leave the thymus, and the comparative lack of lymph node seeking ability conforms to the postulate that they are not functionally as mature as lymph node cells.

Spleen cells are a heterogeneous population and there are several components which have been shown not to be lymph node seeking, such as the haemopoietic precursors and rapidly dividing cells (Zatz & Lance, 1970). The components of the spleen which do reach the lymph nodes are probably the recirculating  $T_2$  and  $B_2$  cells.

The low localisation of thymocytes within the lymph nodes of recipients is reasonable in view of their immaturity.

#### (11) Spleen

$^{75}\text{Se}$  labelled lymph node cells, cortisone resistant thymocytes, spleen cells and thymocytes appear to localise in the spleen in lesser quantities than do similar cells labelled with  $^{51}\text{Cr}$  (Zatz & Lance, 1970; Blomgren & Andersson, 1972). Possible explanations are that  $^{75}\text{Se}$  may fail to label or damage some spleen seeking population and that  $^{51}\text{Cr}$  may overestimate splenic localisation by virtue of being reutilised in the spleen or by altering membrane receptors and diverting cells to the spleen.

The only spleen seeking populations that the above cell suspensions have in common are immature T cells and dividing T cells. The localisation of  $^{75}\text{Se}$  labelled cortisone resistant thymocytes to lymph nodes was similar to that of similar cells labelled with  $^{51}\text{Cr}$  (Blomgren & Andersson, 1972) suggesting immature cells are not damaged by  $^{75}\text{Se}$ . Lymph node cells and thymocytes which were labelled with  $^{125}\text{I}$ UdR were found to migrate to the spleen (Zatz & Lance, 1970), however the autoradiographic data with  $^3\text{H}$ -methionine (Fig. IV.4) suggests that dividing cells are labelled with  $^{75}\text{Se}$ , and the ability of labelled cells to proliferate to



oxazolone (Fig. IV.5) suggests (though does not prove) that blast cells are not damaged by the isotope. 95% of thymocytes are immature cells which are not normally found in the circulation, and the finding that 24.8% of the injected dose, labelled with  $^{51}\text{Cr}$ , was found in the spleen 24 hrs after their injection (Zatz & Lance, 1970) suggests that their presence in the spleen may not be immunologically significant. Lack of information about the possible reutilisation of  $^{51}\text{Cr}$  in the spleen (by red cells or macrophages) precludes any definite conclusions about these discrepant results.

(iii) The apparent movement of lymphocytes from spleen to lymph nodes between 6 and 24 hrs.

This phenomenon, apparent from the results described in Table IV.10 and Fig. IV.8 was also shown to occur using  $^{51}\text{Cr}$  labelled lymph node cells (Zatz & Lance, 1970; Bainbridge et al., 1966) and mouse thoracic duct cells (Sprent, 1973). There is strong evidence to suggest that it can be explained in terms of lymphocytes leaving the spleen after about 6 hrs and a proportion of them seeking the lymph nodes. The evidence of an intrasplenic pool of recirculating T lymphocytes that have a spleen transit time of 4 to 6 hrs has been established (Ford, 1972). However, the present figures demonstrate that a proportion (3-8% of the inoculum) leave the spleen after 6 hrs and were thereafter not accounted for. It is possible that these were damaged cells which were trapped in the spleen, phagocytosed, and the label consequently released.

(b) The significance of activity recovered from itssues other than the lymph nodes and spleen

The source of radioactivity recovered from the liver, femur, lungs,

thymus, blood, intestine and kidneys can be ascertained by comparison of the localisation of dead cells or supernatant with that of live cells.

(i) Liver

That localisation in the liver is not immunologically significant is suggested by the finding that there was no difference in the amount of localisation by dead cells or live cells (Table IV.5). It has been shown that the removal of lymphocyte glycoproteins results in a decrease in the number of cells that localise to lymphoid tissue with a concomitant increase in the liver (Woodruff & Gesner, 1968, 1969). This suggests that cells which do not normally circulate, such as dead, dying, damaged or possibly dividing cells, are sequestered by the liver. In view of the reported recovery of label from the liver of recipients of heat killed  $^{51}\text{Cr}$  labelled cells (Bainbridge *et al.*, 1966) and thoracic duct cells labelled with high concentrations of  $^{51}\text{Cr}$  (K. Donald, unpublished results) it was expected to find that an increase would be observed in the liver recovery from recipients of frozen/thawed or allogeneic cells (Tables IV.5 and IV.8). The fact that this was not found may be due to the rapid destruction by the liver of the dead cells and excretion of the label. That the free amino acid,  $^{75}\text{Se}$  can be incorporated by liver cells is suggested by the finding that 9.5% of the supernatant counts were found in the liver and the report by Waterlow *et al.* (1969) that recycling of  $^{75}\text{Se}$  occurs in the liver.

(ii) Femur

A range of 0.4-2.6% of the injected dose was recovered from the femurs of animals which had been injected with  $^{75}\text{Se}$  labelled cells, a similar

recovery was obtained using  $^{51}\text{Cr}$  (Fig. IV.14). The mice had not been perfused and therefore a proportion of the radioactivity may be due to the blood content of the bones. Experiments to test the functional capacity of bone-marrow cells have demonstrated the presence of T cells (Doenhoff et al., 1970) and although more activity was recovered from the femurs of recipients of lymph node lymphocytes and cortisone resistant thymocytes than of thymocytes, it seems unlikely a cell-localisation technique could detect migration of mature T cells to the bone-marrow.

#### (111) Lungs

Significantly lower amounts of radioactivity were recovered from the lungs of mice that had been injected with supernatant than from mice which had received live lymph node cells (Table IV.4), suggesting that recovery in the lungs does represent labelled cells. The present studies showed two interesting phenomena regarding localisation in the lungs; the first was the enhanced localisation of dead cells at 24 and 72 hrs compared to live cells (Table IV.5) and the second was the tendency to obtain a greater recovery of label at 2 and 6 hrs than at 24 and 72 hrs (Tables IV.11, 12 and 14). These results suggest that the lungs are the first trapping network to be encountered by intravenously injected cells and that dead cells, by virtue of their immobility, are probably less able to escape this trap than live cells. That lymphocytes become transiently caught in the lungs has been suggested by the results of several authors (Weisberger et al., 1951; Mims, 1962; Hall, 1967; Halstead & Hall, 1972; Hall et al., 1972). Hall et al. (1972) using autoradiography, revealed the presence of  $^3\text{H}$ -thymidine labelled thoracic duct cells in the alveoli and capillaries of lungs.

(iv) Thymus

Recovery of  $^{75}\text{Se}$  from the thymus varied from about 0.1-0.7% of the injected dose. The amount of activity which was recovered from the thymus of mice which had received supernatant or frozen/thawed cells was very variable (Table IV.4 and Table IV.5), but the failure to detect any difference in apparent thymic localisation of syngeneic or allogeneic cells (Tables IV.8 and 9) suggests the activity does not emanate from cells that entered the thymus. There is little evidence that mature lymphocytes enter the thymus (Micklem et al., 1966) although autoradiographic studies have detected some cells in the thymus of rats which received  $^3\text{H}$ -uridine labelled thoracic duct cells (G. Rannie, pers. comm.). The major proportion of the radioactivity found in the thymus may be due to the blood content, and reutilisation of label disseminated by the blood. Virtually no  $^{51}\text{Cr}$  activity was recovered from the thymus of mice which had received  $^{51}\text{Cr}$ -labelled spleen cells until 72 hrs after the injection of dead cells (Table IV.14). This low level of recovery is supported by reports from other authors using  $^{51}\text{Cr}$  (Bainbridge et al., 1966; Sprent, 1973).

(v) Blood

The amount of radioactivity recovered in the blood varied from 3-10% in 1 ml. Evidence precludes the possibility that this represents labelled cells. The amount of activity recovered in the blood of mice injected with supernatant (Table IV.4) was not less than that recovered from mice which had been given live cells. It is difficult to predict the levels of activity which might be expected from the blood of recipients of dead cells, for although they would lack recirculating healthy cells, they

may have a surplus of dead cells that cannot localise. This may explain why there was no difference in the recovery of activity from blood of recipients of frozen/thawed or live cells (Table IV.5). In 2 experiments (Table IV.11 and IV.5) the blood was centrifuged before counting and the plasma was removed. The plasma consistently contained more counts than the red cells, sometimes by a factor of 3. Samples of plasma were chromatographed on a Sephadex G200 gel filtration column and the major part of the activity was found to be associated with the serum protein fraction as opposed to the free amino-acid. From the point of view of reutilisation of eluted radioactivity, it may be more advantageous for the isotope to be in the form of serum proteins. The existence of  $^{75}\text{Se}$  in serum proteins may be due to the ability of the liver to metabolise the free amino-acid (Waterlow et al., 1969) which possibly elutes from labelled cells.

The recovery of  $^{51}\text{Cr}$  from blood was very low (Table IV.14), varying from 0.1-0.4% of the injected dose. This compares to a recovery of 1.5% in 1 ml from mice which received  $^{51}\text{Cr}$  labelled lymph node cells (Zatz & Lance, 1970) and thoracic duct cells (Sprent, 1973). The results in these studies (Table IV.14) show that the major part of the activity is found in the plasma. It is curious that in experiments which utilised  $^{51}\text{Cr}$ -labelled rat thoracic duct cells, as much as 6% of the injected dose was found in the plasma (K. Donald, unpublished results). The discrepancy may be due to different labelling conditions.

#### (vi) Intestine

The recovery of activity from the intestine of mice injected with

<sup>75</sup>Se-labelled spleen cells was high (11% of the injected dose) but probably does not represent labelled cells. Thus there was no difference in the recovery of label from the intestine of recipients of live and dead cells (Table IV.5) and the propensity for intestinal epithelium to incorporate <sup>75</sup>Se has been demonstrated (Hansson & Jacobsson, 1966). Preliminary experiments indicated that very low levels of activity are recovered from the Peyer's patches. The source of label in the intestine was probably elutate. Large blast like lymphocytes, particularly B cells appear to be the major cell type that localise to the gut (Hall *et al.*, 1972; Guy-Grand *et al.*, 1974; Parrott & Ferguson, 1974).

(vii) Kidneys

Kidneys do not appear to perform any immunological function and when healthy only contain lymphocytes present in the blood. <sup>75</sup>Se activity disappears from the body quite rapidly as was seen in the total activity which was recovered from mice given thymocytes or supernatant. Presumably a large proportion of label is eliminated through the kidneys, although this is not apparent in terms of activity recovered from these organs.

(viii) Total recovery of radioactivity

One of the perplexing aspects of these experiments, and indeed any experiment utilising labelled cells, is the depressing rapidity with which label disappears from the whole animal. The disappearance is apparent from the poor recovery obtained from all the organs counted compared with the activity which was injected. A proportion of injected

radioactivity may disappear very rapidly, for example Hall et al. (1972) recovered 70% of the activity from  $^{125}\text{IUdR}$  or  $^3\text{H}$ -thymidine labelled blast cells 30 mins after intravenous injection, this may imply that cell death and catabolism of fragile cells occurs. The loss of activity from lymph nodes and spleen over 5 days (Fig. IV.8) represents metabolic turnover of proteins containing  $^{75}\text{Se}$  and dying cells. Whole body counts were not performed on the animals (Fig. IV.8) and it is only presumed that the activity was excreted. If the activity was utilised by any other tissues, the rapidly metabolising liver and intestinal epithelium would be obvious locations, and the results from Fig. IV.8 and Table IV.5 suggest this does not happen.

The amount of label recovered from an animal decreases with time after the injection of the cells. Less activity was recovered from the selected organs at 24 hrs than at 6 hrs (Tables IV.10, 11 and 12) and at 72 hrs than at 24 hrs (Table IV.5). The amount of recoverable activity varied with the cell suspension. Low recovery appears to be generally associated with an inability of cells to localise in lymphoid tissue and spleen, and apart from a transient increase in the lung, a concomitant increase in activity is not detected in any other organs.

These results stimulate interest in the role which the liver plays in eliminating lymphocytes that do not localise in lymphoid tissue, spleen or intestine. Dead, damaged, allogeneic and possibly some large dividing cells are catabolised and a major proportion of the label is excreted. Presumably, in the absence of direct evidence from these studies, they are catabolised by the liver. Studies on the membrane

changes that occur to allow sequestration of circulating cells by the liver, may contribute to knowledge about the structures of surface components necessary for lymphoid localisation.

#### 8. Localisation of rat thoracic duct cells

Only 3% of labelled thoracic duct cells were recovered from the liver of syngeneic recipients. This indicates that the fairly high liver recovery in the present studies utilising mice was a function of the type of cell injected rather than an indication that  $^{75}\text{Se}$  damages cells. The results showed a similar distribution to the spleen and lymph nodes as were found using mice. The blood contained less activity than was found in the blood of mice and may be a consequence of the inoculation of an undamaged naturally circulating population of cells.

#### 9. General comments

A summary of the properties of lymphocytes labelled with  $^{75}\text{Se}$  is as follows:

- a)  $2\mu\text{Ci}/10^7$  cells produces an average of 100,000-200,000 cpm.
- b) Lymph node lymphocytes, thymocytes, cortisone resistant thymocytes, spleen cells and bone-marrow cells all incorporate the isotope. Evidence obtained using  $^3\text{H}$  methionine suggests that there is no difference in incorporation between T and B cells, but there is a direct correlation between incorporation and cell size.
- c) 75% of the incorporated isotope is found in the TCA precipitable fraction of cells. The rate of elution in vitro is about 50% over 24 hrs.



d) Reutilisation studies suggest that about 1% of label released from injected dead and dying cells, cells killed in situ and label from live cells will remain in lymph nodes or recipients and 2% may be found in the spleen.

e)  $^{75}\text{Se}$  does not appear to damage the ability of lymphocytes to localise and proliferate in response to oxazolone, nor to recognise sheep erythrocytes, and cooperate to produce  $\gamma\text{M}$  and  $\gamma\text{G}$  antibody.

f) Localisation studies suggest that this label is suitable for the detection of localisation in lymph nodes. It may be suitable for studying localisation in the spleen, but the results are not the same as those obtained with  $^{51}\text{Cr}$ . Activity recovered in the blood, liver and thymus reveals little about the immunological activity of these organs.

The salient point about these studies is that certain features of the label (it elutes, does not label cells uniformly and is partially reutilised in vivo) impose restrictions on its use and complicate the interpretation of the results. The label is most useful for studying localisation in situations where large amounts of activity are expected to be recovered, for example in the lymph nodes. There are certain experimental situations where results using  $^{75}\text{Se}$  would have to be interpreted with great caution and confirmed with autoradiography.

These are:

- (i) When studying localisation in non-lymphoid tissue.
- (ii) When comparing the localisation of different cell types, because of the different incorporation by cells of different sizes.

These restrictions apply to every isotope at present used to study cell localisation, with the possible exception of  $^{125}\text{IUdR}$  which has other disadvantages, and  $\text{Tc}^{99\text{m}}$  about which very little is known.

Dr. Gordon Rannie kindly compiled a table (Table IV.15) comparing the activities recovered in various organs of rats injected with thoracic duct cells labelled with  $^3\text{H}$ -uridine,  $^{14}\text{C}$ -uridine,  $^{75}\text{Se}$ ,  $^3\text{H}$ -leucine and  $^{14}\text{C}$ -leucine. The important feature is the occurrence of high levels of activity in the plasma is not restricted to  $^{75}\text{Se}$  labelled cells, and that 23.4% of the injected  $^3\text{H}$ -uridine and 32.14% of the injected  $^3\text{H}$ -leucine was recovered in the perfusate of the recipients. These high levels demonstrate the need for caution in interpreting quantitative results from tissues where the blood could be contributing a major portion of the activity.

These studies have defined some of the parameters of use of another emitting isotope which has the useful features that it labels all lymphocytes (as opposed to only dividing cells), does not appear to damage immunocompetence, and could provide a useful comparison with results obtained with other isotopes.

## CHAPTER V

### CELL RECRUITMENT DURING THE RESPONSE TO OXAZOLONE

#### RESULTS

Experiments were designed to measure the accumulation of labelled lymphocytes in the regional lymph nodes of mice, 1-5 days after the application of oxazolone. The experiments had 2 objectives:

- (a) To investigate the relationship, if any, between the amount of proliferation occurring within a lymph node and the ability of that lymph node to recruit and retain circulating lymphocytes.
- (b) To investigate the kinetics of cell localisation in antigen-stimulated lymph nodes from 1-5 days after administration of the antigen.

Mice which had been lethally irradiated were injected with  $10^6$  or  $10^8$  thymocytes, or with BSS. On the same day they were painted with either oxazolone or alcohol. In this way, 3 groups of mice were established, those giving a 'heavy' proliferative response with  $10^8$  thymocytes, those giving a 'moderate' response with  $10^6$  thymocytes and those giving little response with BSS only. The accumulation of labelled lymph node lymphocytes in the draining and contralateral lymph nodes of these mice was measured 1,2,3,4 and 5 days after oxazolone was administered.

Two experiments were performed, one utilised  $^{75}\text{Se}$  and the other utilised  $^3\text{H}$ -leucine.

### 1. Experiment utilising $^{75}\text{Sem}$

The proliferative response of lymph nodes of experimental mice was measured by the incorporation of  $^{125}\text{IUdR}$  and correlated with the accumulation of  $^{75}\text{Sem}$ -labelled lymph node lymphocytes 1,2,3,4 and 5 days after the administration of oxazolone. The experimental protocol is shown in Fig. V.1.

Oxazolone was painted on the hind foot pad to localise the response to the draining popliteal and inguinal nodes and avoid involvement of the spleen and distant lymph nodes (Chapter II). The mice were sacrificed 24 hrs after they received  $^{75}\text{Sem}$  labelled cells and 2 hrs after they had received  $^{125}\text{IUdR}$ . Four lymph nodes were removed from each animal; the draining and contralateral popliteal and inguinal nodes. They were counted individually to assess their content of  $^{75}\text{Sem}$  and  $^{125}\text{IUdR}$ .

The incorporation of  $^{125}\text{IUdR}$  by the inguinal nodes of experimental mice are shown in Fig. V.2 and V.3. The amount of  $^{125}\text{IUdR}$  incorporated by mice which had received  $10^8$  thymocytes and oxazolone increased between days 2 and 4 and declined between days 4 and 6. (Fig. V.2). The response of the mice which had received alcohol and  $10^8$  thymocytes increased gradually between days 2 and 6. The incorporation of  $^{125}\text{IUdR}$  by recipients of  $10^6$  thymocytes (Fig. V.3) shows a contrasting pattern. There was little difference between the proliferative response of recipients of oxazolone and of alcohol between days 2 and 5, and only on day 6 is there an appreciable response to oxazolone. The amount of  $^{125}\text{IUdR}$  incorporated by the inguinal nodes of saline-injected mice is shown in Fig. V.2. A low level of incorporation was found between days 2 and 5, and

the increase found on day 6 may have been due to some recovery of the host's lymphocytes.

The objective of this experiment was to compare the accumulation of labelled lymphocytes in the lymph nodes of irradiated mice reconstituted with  $10^6$ ,  $10^8$  thymocytes or saline, and undergoing a 1,2,3,4 or 5 day response to oxazolone. The experimental protocol ensured that all groups received labelled cells on the same day, from the same cell suspension; therefore a direct comparison between the  $^{75}\text{Se}$  activity found in the lymph nodes of the various groups was possible. The results are expressed as the  $\log_{10}$  of the mean 2-minute counts.

The localisations of  $^{75}\text{Se}$  labelled lymphocytes in the inguinal nodes of the various groups is shown in Figs. V.4-V.6. Although the popliteal nodes were counted, only the results from those mice which received  $10^8$  thymocytes are presented (Fig. V.7). The variation found in the popliteals of recipients of  $10^6$  thymocytes or BSS were too large to allow interpretation of the results. Essentially there are no obvious differences in the pattern of cell-localisation between groups that received different doses of thymocytes. Thus it appears from these results that cellular proliferation has not affected the magnitude or kinetics of cell-localisation in response to oxazolone, over the time period studied. There emerges from these figures a pattern of localisation, the major features of which are as follows:

- (a) One day after oxazolone administration the amount of  $^{75}\text{Se}$  activity found in the regional lymph nodes was significantly greater than that found in the contralateral nodes (Figs. V.4, V.5, V.6 and V.7) and

significantly greater than that found in lymph nodes of mice that received alcohol (Figs. V.4, V.6 and V.7).

(b) The greatest amount of cell-localisation in terms of absolute counts was found 1 day after antigen administration. The counts which were obtained from the 3 groups at this time were almost identical.

(c) Two days after oxazolone administration the amount of localisation of labelled cells in the regional lymph nodes fell significantly below the day 1 level (Figs. V.4, V.6 and V.7) to a level similar to (Fig. V.5) or below (Fig. V.4, V.6 and V.7) that found in the lymph nodes of animals treated with alcohol. In Figs. V.4 and V.6 this decrease was not confined to the draining lymph nodes and was also found in the contralateral lymph nodes.

(d) On days 3 and 4 the amount of activity found in the lymph nodes draining the site of oxazolone application was greater than that found in the contralateral nodes of the same recipients, although this difference was not significant at day 4 (Fig. V.4 and V.7). By day 5 there was no significant difference in the localisation of labelled cells to lymph nodes draining oxazolone and the respective contralateral lymph nodes (Figs. V.4 and V.5). In Fig. V.6, however, localisation was still apparent in the draining compared to the contralateral nodes at day 5. In Fig. V.7 no data for localisation in the contralateral nodes at day 5 are available.

(e) The amount of activity found in the lymph nodes draining the site of antigen application was on each day (except day 5, Fig. V.5) greater than that found in the contralateral nodes of the same animal. In contrast, the mice which received alcohol showed similar levels of activity in their draining and contralateral lymph nodes.

## 2. Experiment utilising $^3\text{H}$ -leucine

The experimental protocol used was as described for  $^{75}\text{Se}$ , except that  $^3\text{H}$ -leucine was used to label lymphocytes and DNA synthesis was not estimated. The excised lymph nodes were individually homogenised in 10% TCA, filtered and the precipitate was counted in a liquid scintillation counter. The results are expressed as the  $\log_{10}$  of the mean 2-minute counts. The variation found with the results from the activity counted in the inguinal nodes was too large to allow interpretation of the results. The localisation of  $^3\text{H}$ -leucine labelled lymphocytes in the popliteal lymph nodes of the various groups is shown in Figs. V.8, V.9 and V.10.

There was no significant difference in the amount of localisation which was found in the lymph nodes of mice which were undergoing a proliferative response to oxazolone, and the lymph nodes of mice which had received BSS only. Thus in accordance with the results obtained using  $^{75}\text{Se}$ , cellular proliferation did not appear to affect cell recruitment. These results do not however exhibit all the features of cell localisation to oxazolone which were found using  $^{75}\text{Se}$ . In particular 2 previously observed features were not discerned here:

(a) Using  $^{75}\text{Se}$ , maximum localisation in terms of absolute counts was found 1 day after oxazolone administration. The present results show no more activity in the draining popliteals 1 day after oxazolone than at 2 and 3 days after oxazolone.

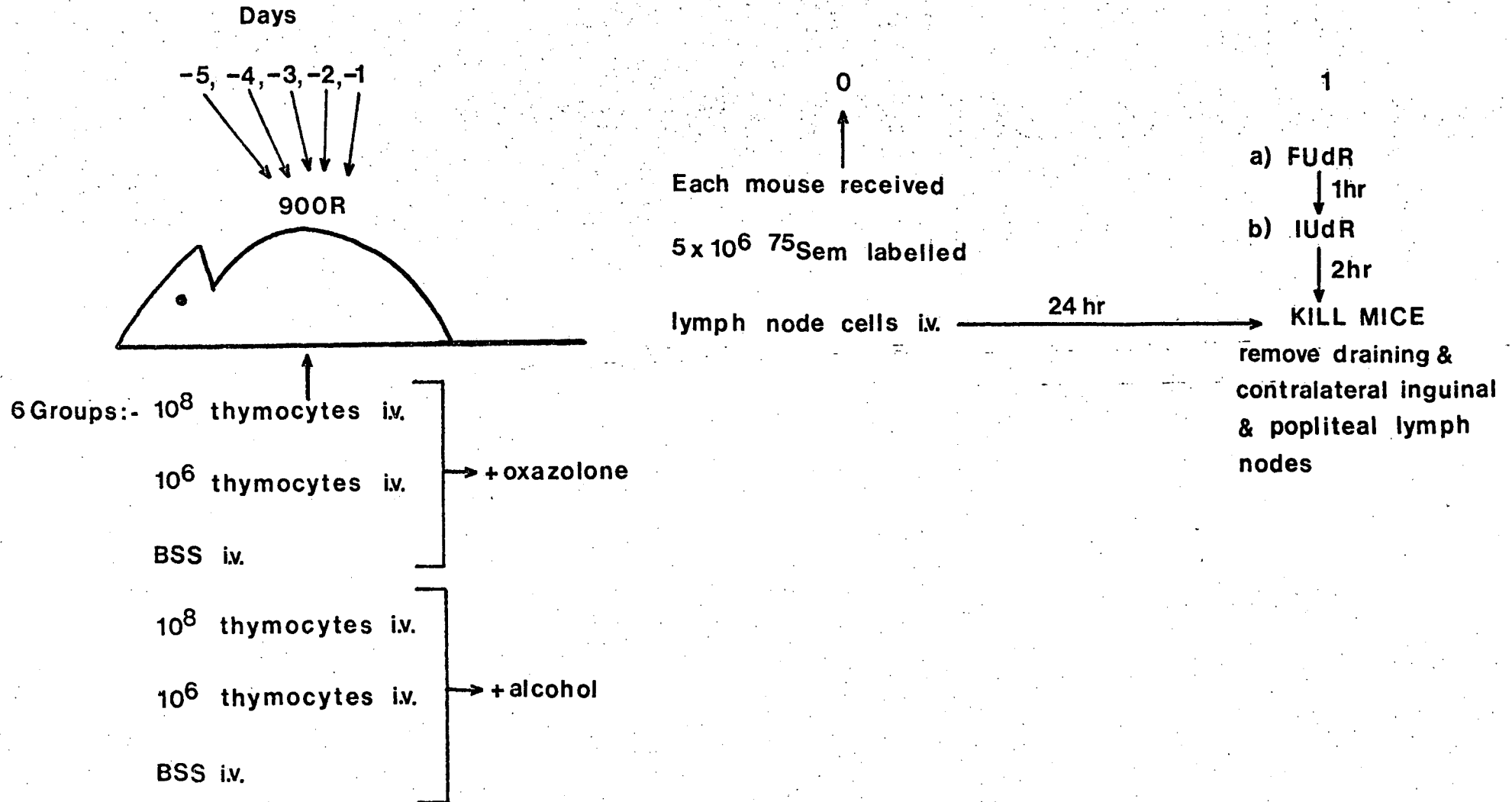
(b) Using  $^{75}\text{Se}$ , there was little difference in the amount of activity detected between the draining and contralateral nodes of recipients of alcohol, 1-3 days after receiving alcohol. The

similarity of the counts obtained between these nodes is an important control and any deviation from their parity indicates an alteration in cell localisation. The interpretation of some of the results using  $^3\text{H}$ -leucine is therefore hindered by the knowledge that there was a significant difference in the localisation of cells between the draining and contralateral nodes of alcohol-stimulated mice at 3 days (Fig. V.8 and V.9). This renders equivocal the apparent significant difference in localisation found between the draining and contralateral nodes of oxazolone-stimulated mice on day 3.

There was, however, significantly more activity recovered from the draining lymph nodes of oxazolone-stimulated mice than the contralateral nodes, on days 1, 2 and 4 (Fig. V.9) and the first 4 days in Fig. V.10 and day 2 in Fig. V.8. This was not matched by an equivalent difference between the draining and contralateral nodes in the alcohol-stimulated mice.



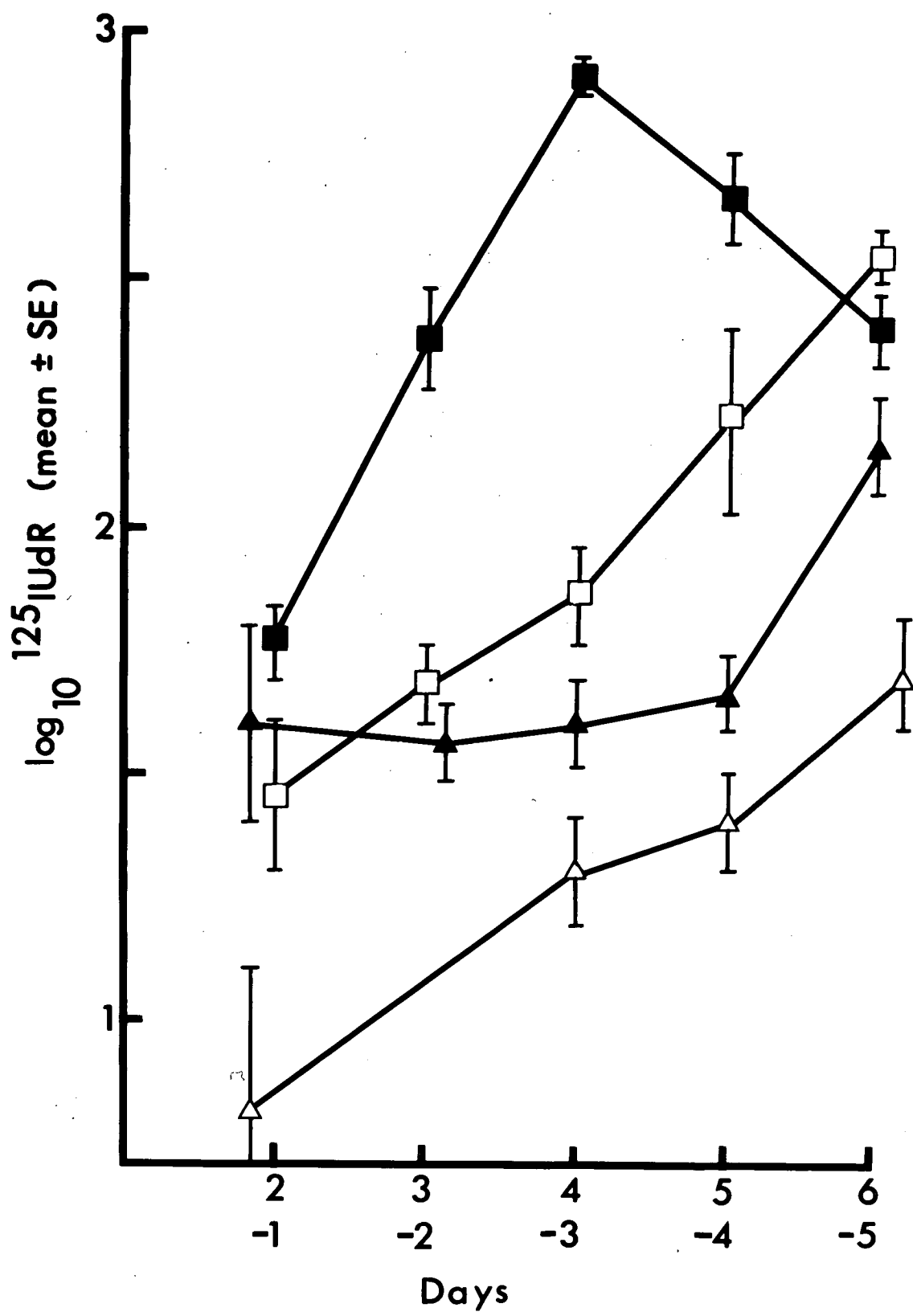
Fig.V.1. Experimental procedure



**Fig. V.2**

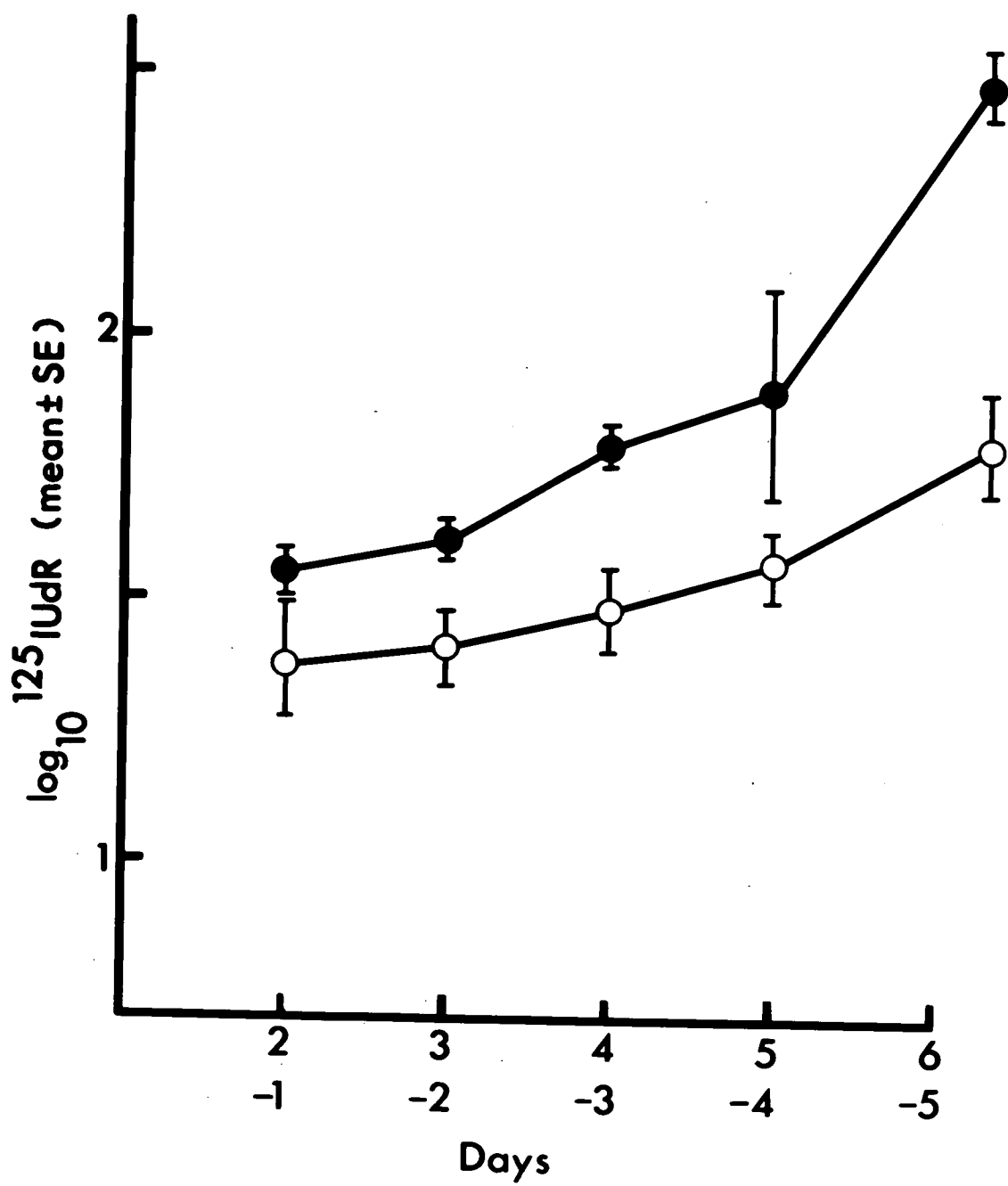
**$^{125}$ IUdR incorporation by the inguinal lymph nodes of mice which had been irradiated (900R) and given intravenous injections of  $10^8$  thymocytes or BSS, 2-6 days after receiving oxazolone or alcohol by foot-pad application.**

- recipients of  $10^8$  thymocytes and oxazolone**
- recipients of  $10^8$  thymocytes and alcohol**
- ▲ recipients of BSS and oxazolone**
- △ recipients of BSS and alcohol**



**Fig. V.2**

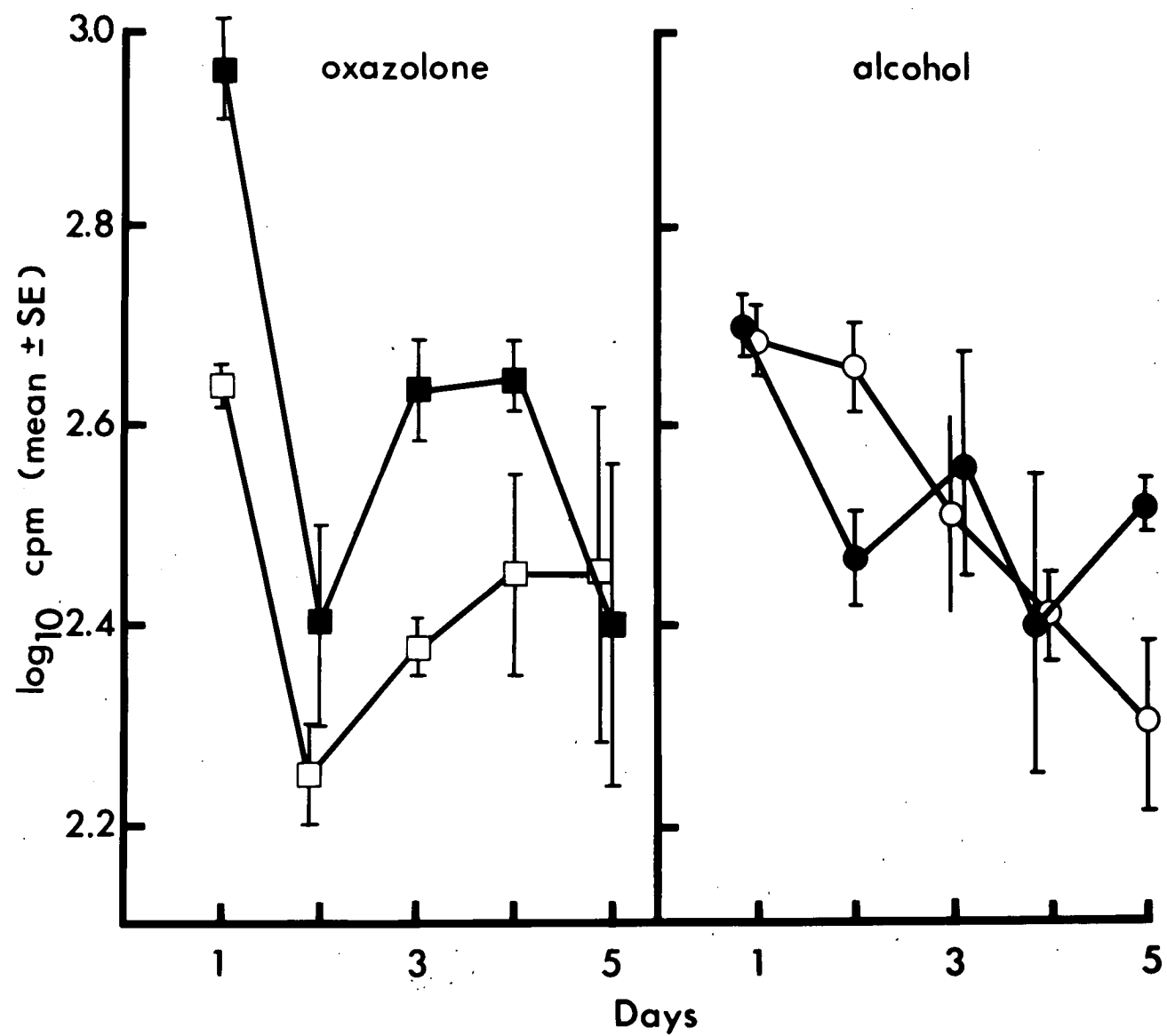
**<sup>125</sup>IUdR incorporation by the inguinal lymph nodes of mice which had been irradiated (900R) and given intravenous injections of 10<sup>6</sup> thymocytes, 2-6 days after receiving oxazolone ● , or alcohol ○ , by foot-pad application.**



**Fig. V.4**

**Localisation of  $^{75}\text{Se}$  labelled lymph node lymphocytes in the inguinal nodes of mice which had been irradiated (900R) and injected with BSS, 1-5 days after receiving oxazolone or alcohol by foot-pad application.**

- $^{75}\text{Se}$  activity in the draining nodes of recipients of oxazolone
- $^{75}\text{Se}$  activity in the contralateral nodes of recipients of oxazolone
- $^{75}\text{Se}$  activity in the draining nodes of recipients of alcohol
- $^{75}\text{Se}$  activity in the contralateral nodes of recipients of alcohol.



**Fig. V.5**

**Localisation of  $^{75}\text{Se}$  labelled lymph node lymphocytes in the inguinal nodes of mice which had been irradiated (900R) and injected with  $10^6$  thymocytes, 1-5 days after receiving oxazolone or alcohol by foot-pad application.**

- $^{75}\text{Se}$  activity in draining nodes of recipients of oxazolone**
- $^{75}\text{Se}$  activity in contralateral nodes of recipients of oxazolone**
- $^{75}\text{Se}$  activity in draining nodes of recipients of alcohol**
- $^{75}\text{Se}$  activity in contralateral nodes of recipients of alcohol.**



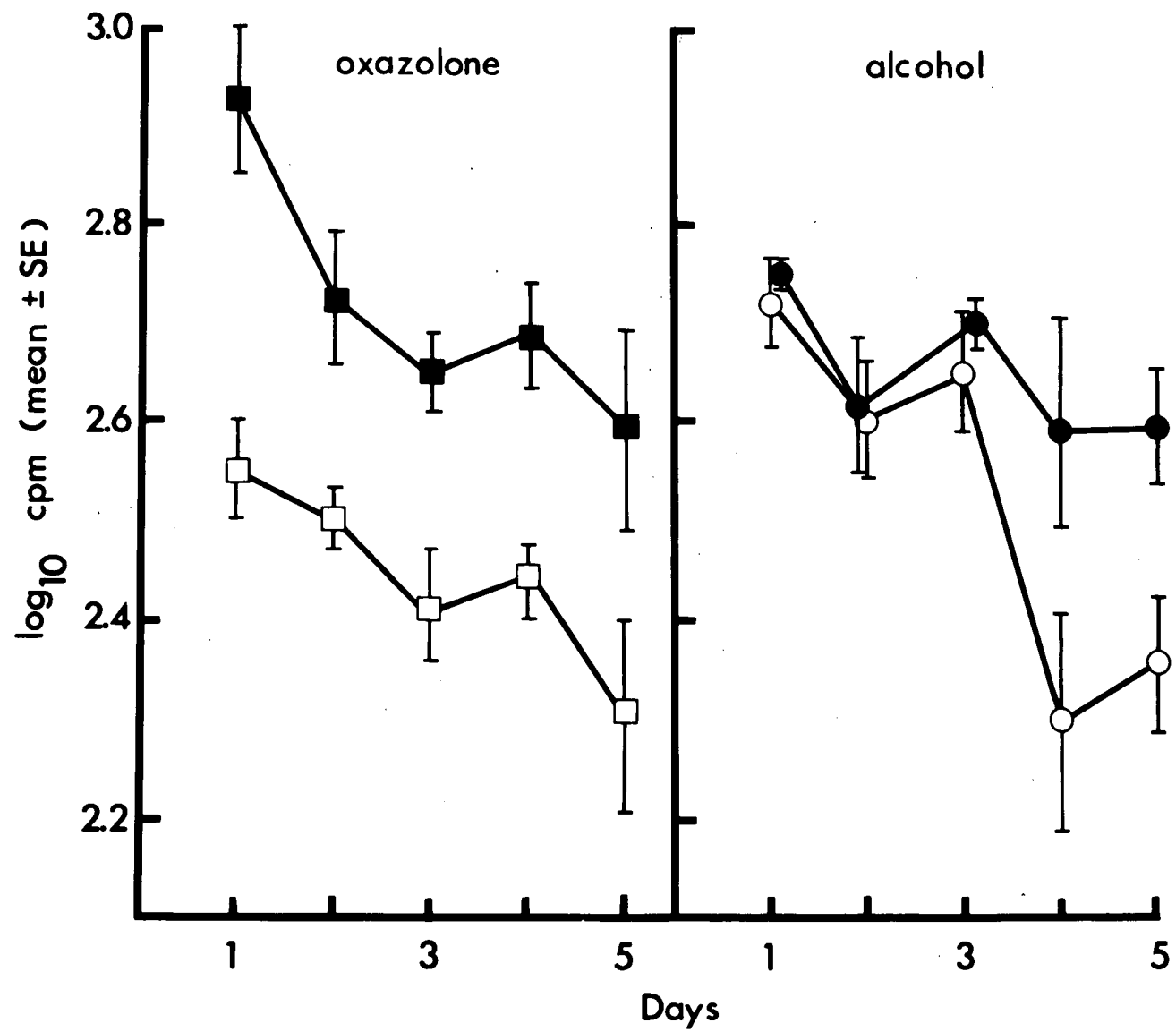


Fig. V.6

Localisation of  $^{75}\text{Se}$  labelled lymph node lymphocytes in the inguinal nodes of mice which had been irradiated (900R) and injected with  $10^8$  thymocytes, 1-5 days after receiving oxazolone or alcohol by foot-pad application.

- $^{75}\text{Se}$  activity in draining nodes of recipients of oxazolone
- $^{75}\text{Se}$  activity in contralateral nodes of recipients of oxazolone
- $^{75}\text{Se}$  activity in draining nodes of recipients of alcohol
- $^{75}\text{Se}$  activity in contralateral nodes of recipients of alcohol

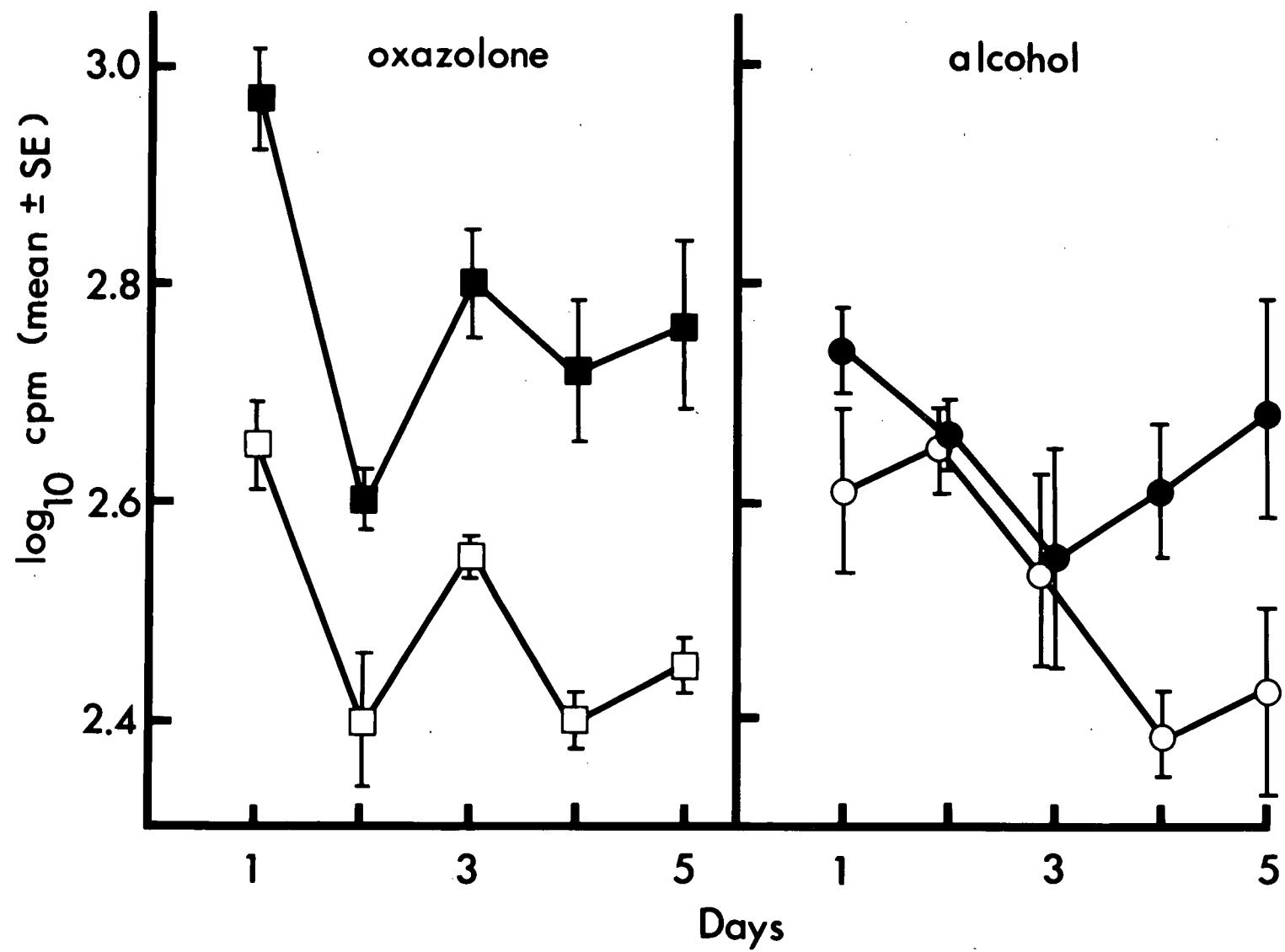


Fig. V.7

Localisation of  $^{75}\text{Se}$  labelled lymph node lymphocytes in the popliteal nodes of mice which had been irradiated (900R) and injected with  $10^8$  thymocytes, 1-5 days after receiving oxazolone or alcohol by foot-pad application.

- $^{75}\text{Se}$  activity in draining nodes of recipients of oxazolone
- $^{75}\text{Se}$  activity in contralateral nodes of recipients of oxazolone
- $^{75}\text{Se}$  activity in draining nodes of recipients of alcohol
- $^{75}\text{Se}$  activity in contralateral nodes of recipients of alcohol.

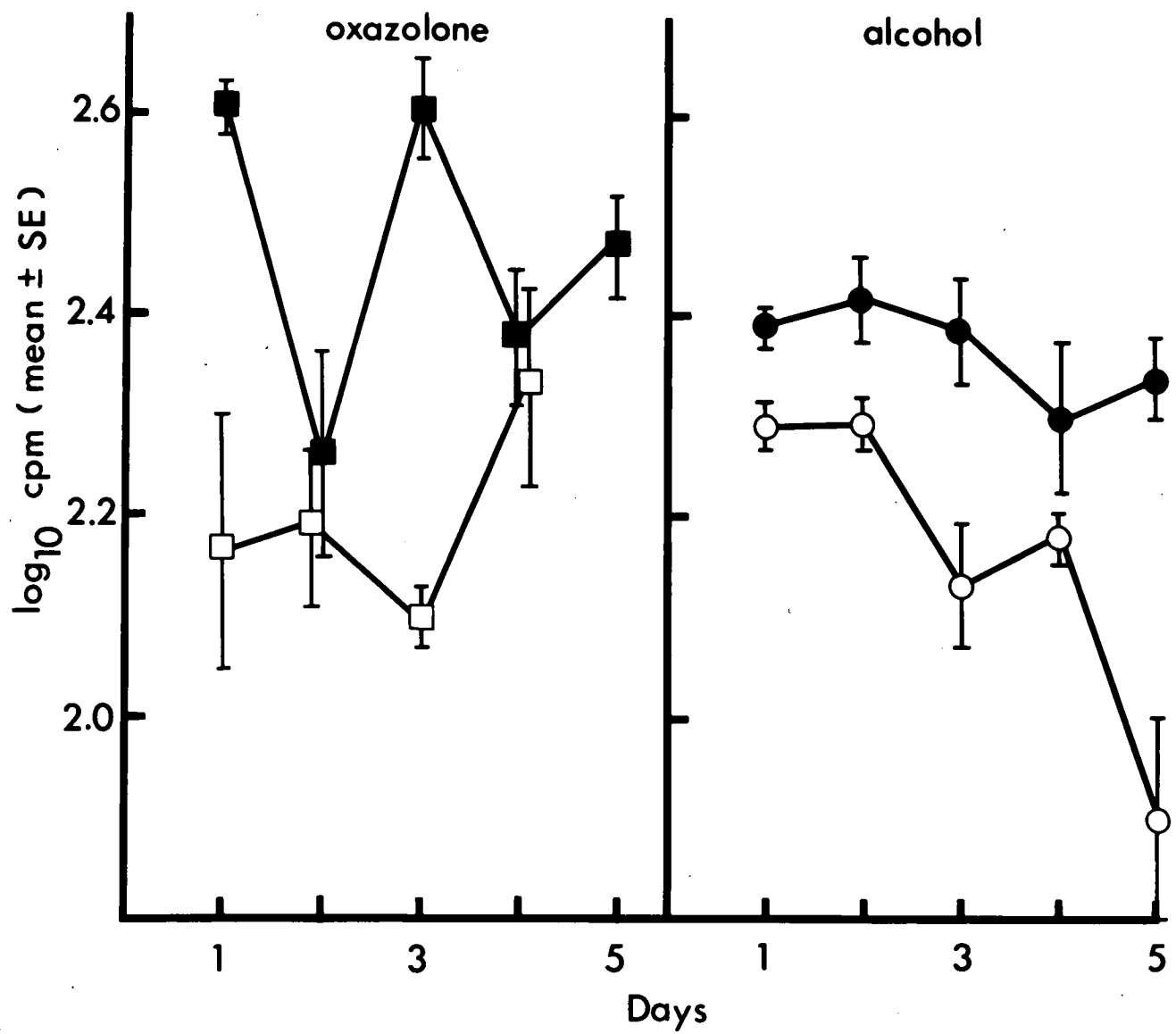


Fig. V.8

Localisation of  $^3\text{H}$ -leucine labelled lymph node lymphocytes  
in the popliteal nodes of mice which had been irradiated (900R)  
and injected with BSS 1-5 days after receiving oxazolone or  
alcohol by foot-pad application.

- $^3\text{H}$  activity in draining nodes of recipients of oxazolone
- $^3\text{H}$  activity in contralateral nodes of recipients of  
oxazolone
- $^3\text{H}$  activity in draining nodes of recipients of alcohol
- $^3\text{H}$  activity in contralateral nodes of recipients of  
alcohol

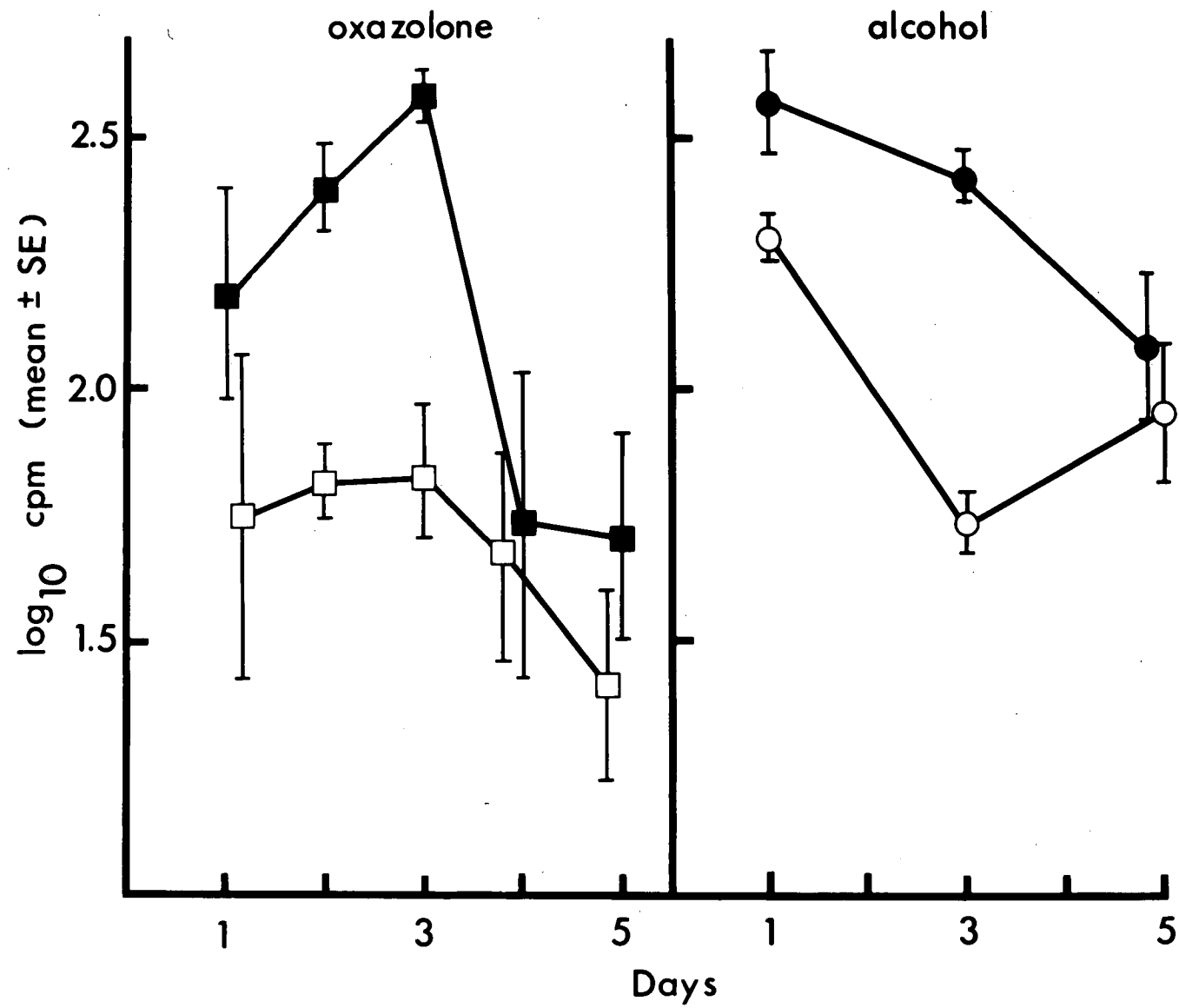


Fig. V.9

Localisation of  $^3\text{H}$ -leucine labelled lymph node lymphocytes in the popliteal nodes of mice which had been irradiated (900R) and injected with  $10^6$  thymocytes, 1-5 days after receiving oxazolone or alcohol by foot-pad application.

- $^3\text{H}$  activity in draining nodes of recipients of oxazolone
- $^3\text{H}$  activity in contralateral nodes of recipients of oxazolone
- $^3\text{H}$  activity in draining nodes of recipients of alcohol
- $^3\text{H}$  activity in contralateral nodes of recipients of alcohol

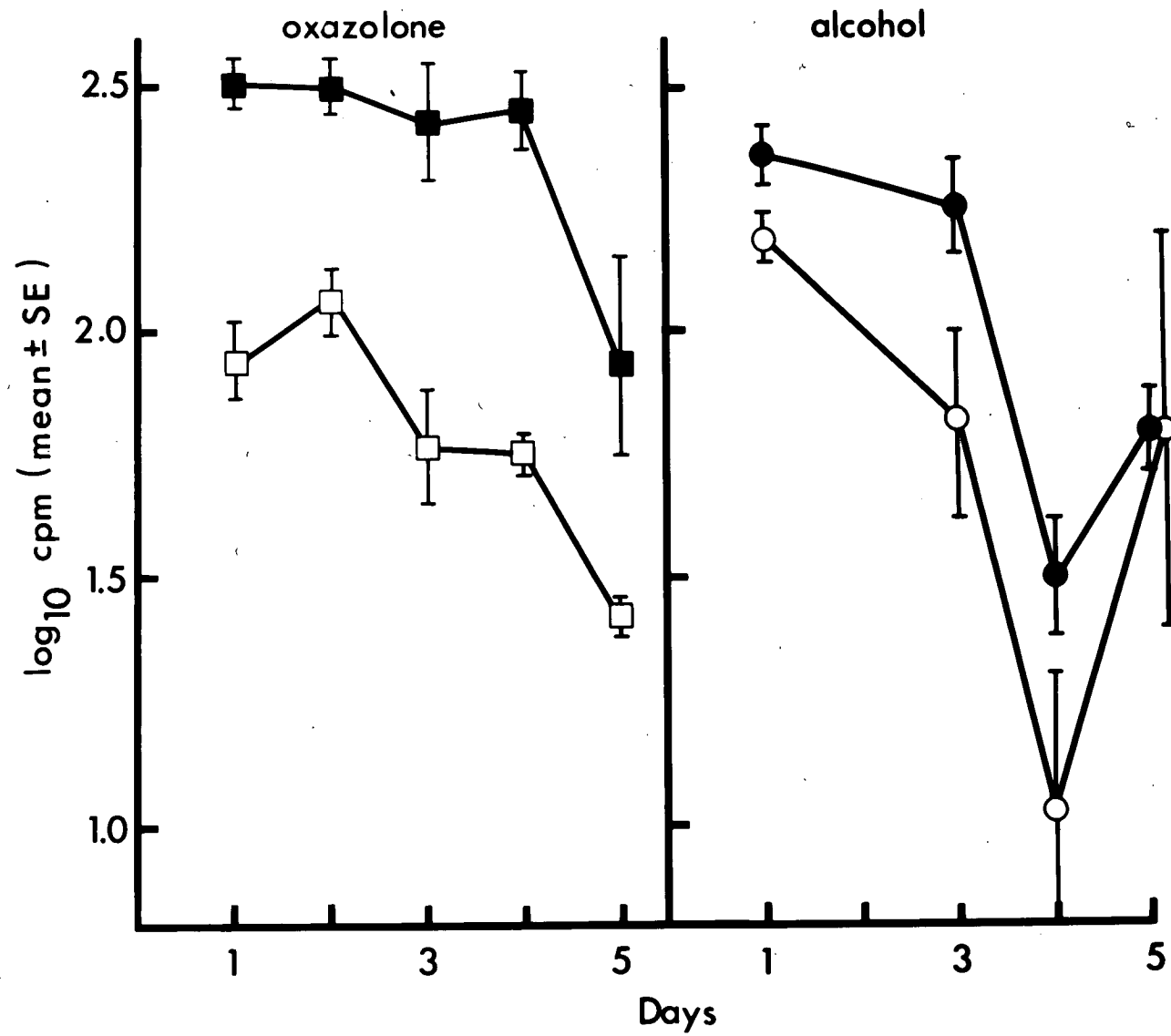




**Fig. V.10**

**Localisation of  $^3\text{H}$ -leucine labelled lymph node lymphocytes in the popliteal nodes of mice which had been irradiated (900R) and injected with  $10^8$  thymocytes, 1-5 days after receiving oxazolone or alcohol by foot-pad application.**

- $^3\text{H}$  activity in draining nodes of recipients of oxazolone**
- $^3\text{H}$  activity in contralateral nodes of recipients of oxazolone**
- $^3\text{H}$  activity in draining nodes of recipients of alcohol**
- $^3\text{H}$  activity in contralateral nodes of recipients of alcohol**



## DISCUSSION

The results of experiments presented in Chapter II suggested the existence of a homeostatic mechanism responsible for regulating the magnitude of the proliferative response of lymphocytes to oxazolone. The mechanism was evidently operative 4-5 days after the administration of oxazolone but may well be in force earlier. It is known that one of the early events in an immune response consists of an antigen-mediated recruitment of circulating cells (Hall & Morris, 1964; Ford & Gowans, 1967; Simic & Petrovic, 1971). Any mechanism which could inhibit recruitment and retention of lymphocytes may deprive lymph nodes of immunocompetent cells, and so impede the progress of the immune response. If this hypothesis is correct, and if it accounts for the pattern of the kinetics of the thymocyte proliferative response to oxazolone which was described in Chapter II, it would be predicted that the antigen-stimulated lymph nodes of mice which received only saline would allow the accumulation of a greater number of lymphocytes than would the lymph nodes of recipients of  $10^8$  thymocytes which were undergoing a proliferative response to oxazolone. The experiments in this section were designed to test this prediction.

In Chapter IV, some of the properties of  $^{75}\text{Se}$  have been described, and it was concluded that  $^{75}\text{Se}$  provides a suitable isotopic marker for lymphocyte localisation studies. The results of an experiment performed with  $^{75}\text{Se}$  were compared to those obtained using  $^3\text{H}$ -leucine to label lymphocytes. The results suggest that  $^{75}\text{Se}$  provides a more sensitive system for quantitating cell localisation because;

- (a) There was little difference between the activity recovered from the draining and contralateral nodes in alcohol-stimulated mice using  $^{75}\text{Se}$ , thus providing a 'control' where little localisation in the draining nodes is expected.
- (b) The features that characterised the kinetics of cell accumulation in response to oxazolone were more consistent using  $^{75}\text{Se}$  than using  $^3\text{H}$ -leucine.

The most likely explanation for the greater efficacy of  $^{75}\text{Se}$ , in this system, is the ease of tissue preparation and consequent reduction of experimental errors. Only the experiments which utilised  $^{75}\text{Se}$  are discussed in the following pages.

#### 1. The effect of cellular proliferation on cell recruitment

Experiments measured the DNA synthetic response to oxazolone in the draining lymph nodes of mice that had been irradiated and injected with  $10^8$  or  $10^6$  thymocytes, or saline. Accumulation of labelled cells reached a peak one day after the administration of oxazolone and thus preceded the peak DNA synthetic response which occurred on day 4 in recipients of  $10^8$  thymocytes and on day 5 in recipients of  $10^6$  thymocytes. Labelled cells accumulated in the lymph nodes of mice which had received BSS only and which failed to produce a proliferative response to oxazolone.

A similar temporal relationship between cell trapping and DNA synthesis was found by Zatz and Gershon (1974), who measured the accumulation of  $^{51}\text{Cr}$  labelled cells in the spleens of mice undergoing a graft vs host response. Despite the vast difference in proliferative activity of the

lymph nodes in the groups of mice given different doses of thymocytes, there was no discernible difference in the magnitude of accumulation of labelled cells in the lymph nodes. The results may tentatively indicate that marginally more cells were recruited by lymph nodes containing  $10^8$  responding cells than lymph nodes containing  $10^6$  or not containing cells, a phenomenon also possibly suggested by the results of Zatz and Gershon (1974). Great caution is, however, required in interpreting this issue as it is possible that a large number of proliferating cells has a greater propensity to reutilise label than a small number of proliferating cells.

It is not known what mechanism regulates the entry of lymphocytes into antigen-stimulated lymph nodes. There is, however, some circumstantial evidence that may favour the existence of a type of T cell that directly or indirectly via factors produced during its proliferative activity, regulates the entry of cells into lymph nodes. The magnitude of an immune response is dependent on the number of cells that enter the spleen (Ford & Gowans, 1967; Simic & Petrovic, 1971), the majority of circulating cells are T cells (Raff, 1969; Raff & Wortis, 1970; Miller & Sprent, 1971) and T cell proliferation precedes many immune responses (Carter et al., 1969). Further evidence is the existence of T cell products that cause cellular distension in the paracortical regions of lymph nodes (Kelly et al., 1972) and the observation by Parrott and de Sousa (1971) that 24 hrs after the injection of labelled thymocytes, more were found in the lymph nodes of neonatally-thymectomised mice than in normal mice.

Nevertheless, the results reported in the present studies must be added to an accumulating body of evidence (Frost & Lance, 1974a; Frost, 1974) that T cells do not have an obvious regulatory effect on cell recruitment. They cannot, however, be entirely excluded from such a role, because of the possibility that labelled T cells initiate their own recruitment. The presence of antigen and its effect on macrophages is probably a more important factor regulating cell traffic through a lymph node or spleen (Frost, 1974).

## 2. The kinetics of cell localisation to lymph nodes in oxazolone-stimulated mice

The effect of antigen which causes the non-specific retardation of cells moving through a stimulated organ, can be measured in 2 ways:

- (a) By measuring the deficit in the number of cells leaving the node in the efferent lymph (Hall & Morris, 1962, 1963, 1965; Ford, 1969).
- (b) By measuring the accumulation of radioactivity in stimulated lymph nodes of animals that have received labelled lymphocytes (Zatz & Lance, 1971a, 1971b; Emeson & Thursh, 1971; Ford, 1972; Thursh & Emeson, 1972; Frost & Lance, 1974a, 1974b).

The second method was utilised in the present studies.

Interpretation of the results depends on identifying the control values. There are two controls to be considered. The first is the level of radioactivity associated with the contralateral lymph nodes in recipients of oxazolone, the second is the amount of activity found in the lymph nodes of recipients of alcohol. It is important that in the mice where no localisation is expected, there should be no significant

differences in the amount of radioactivity found between the draining and contralateral nodes. Indeed, no significant differences were detected between these nodes in recipients of alcohol, 1-3 days after alcohol administration.

The most definitive conclusion to be drawn from these results, using both controls as criteria, is that labelled cells injected 24 hrs after the application of oxazolone accumulated preferentially in lymph nodes draining the site of antigen application. Therefore oxazolone is yet another antigen to be added to the list (Hall & Morris, 1965; Frost & Lance, 1974a, 1974b) of those that elicit cell recruitment. By 24 hrs more than twice as many counts were found in the stimulated nodes than were found in the lymph nodes of mice treated with alcohol, demonstrating a level of preferential accumulation or localisation similar to that detected in other systems (Zatz & Lance, 1971a, 1971b). That the peak accumulation should be detected within 24 hrs of antigen application also accords with results from other systems (Zatz & Lance, 1971a; Ford, 1968; Thursh & Emeson, 1971; Zatz & Gershon, 1974). Preferential accumulation of lymphocytes in oxazolone stimulated nodes appeared to have ceased, both in respect of the deficit in the contralateral nodes, and the amount of accumulation in lymph nodes of recipients of alcohol, by day 5. On days 3 and 4, more cells accumulated in the lymph nodes draining the application site of oxazolone than in the contralateral nodes, but there was little difference between localisation in the nodes draining oxazolone and the equivalent lymph nodes of recipients of alcohol. Interpretation of this phenomenon depends on determining why consistently less cells accumulated in contralateral lymph nodes of recipients of



oxazolone than in the lymph nodes of mice which had been painted with alcohol. Two explanations are proposed:

- (a) Antigen non-specifically retards cell movement through a stimulated node and also causes the specific retention of antigen reactive cells for 24-48 hrs (Sprent et al., 1971; Rowely et al., 1972). If 1-4 days after oxazolone, cell movement through the stimulated node was retarded sufficiently, labelled cells injected during this period might be caught in the draining nodes, and their accumulation in contralateral nodes consequently depressed. Similarly the retention of antigen reactive cells in the draining nodes might deplete the number of circulating cells available to accumulate in the contralateral nodes. The latter phenomenon would imply the existence of a large number of cells in a lymphocyte population able to recognise oxazolone.
- (b) A highly speculative proposition is that during the early phase of cell-recruitment, a humoral factor is released from the draining nodes which inhibits cell entry into the contralateral and possibly more distant nodes, for 1-4 days. Such a factor was postulated by Black (1974) to account for the immunosuppressive effects of pre-emption.

A curious feature found in some of the results was the depressed accumulation of labelled cells in the lymph nodes draining the site of oxazolone application, 2 days after oxazolone. The results are suspect due to the concomitant decrease in localisation found in the contralateral nodes of these mice. It is conceivable that the 'day 2' group received deficient injections of labelled cells. This experiment would have to be repeated before this decline could be treated cautiously as an indication of a regulatory mechanism inhibiting cell recruitment 2 days after oxazolone.

The present experimental findings can be tentatively interpreted in terms of cell movements caused by oxazolone in the following manner:

- (a) Within 24 hrs of antigen, more cells enter the draining lymph nodes, as a possible consequence of increased blood flow, permeability or some other mechanism allowing cells to enter the node. This may be combined with retardation of cells leaving the lymph node.
- (b) After 24 hrs there is no further positive recruitment of cells. Lymphocytes enter the draining node at the same rate that they enter lymph nodes in unstimulated mice. There is, however, a slower transit time, and this may be accompanied by selection and retention of antigen reactive cells.
- (c) The slowing down of cells moving through the lymph nodes described in (a) and (b) above may deprive the circulation of cells that would normally accumulate in contralateral nodes.
- (d) Traffic through the draining lymph nodes returns to normal probably on days 4 and 5. This is accompanied by localisation of cells in contralateral nodes equal to that found in the lymph nodes of unstimulated mice.

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